Role of Protease Activation in Sarcolemma Na^+-K^+-ATPase Activity in the Heart Due to Ischemia-Reperfusion

By

Alison L. Müller

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Physiology

University of Manitoba

Winnipeg

ACKNOWLEDGEMENTS

Starting a new chapter along the path of learning, especially in an unfamiliar city, can be a daunting experience, so I would first and foremost like to thank both of my parents, Ruth and Dr. Nestor Müller, for their enthusiastic encouragement and belief in me. Their unwavering support and love was a crucial component in both pursuing and finishing my Master's. Thank you both for inspiration, words of encouragement, and lots of love! I would also like to thank my brother, Phillip Müller, for being there for me and listening to the adventures that occurred in Winnipeg.

There are many exceptional people that, without whom, this thesis would not have come to fruition. I would like to thank Judith Olfert, Gail McIndless and Dr. Janice Dodd of the Physiology Department for answering my many questions that popped up along this journey. To Nina Aroutiounova, Donald Chapman, and Dr. Vijayan Elimban for their technical expertise who taught me all that I needed to know to get the experiments done and the little tricks that make all the difference. I would especially like to thank Eva Little for her patience, wisdom, and unwavering work ethic.

To my committee members, Drs. Michael Czubryt, Ian Dixon, Darren Freed, Mohammed Moghadasian and Pram Tappia, I express my sincere gratitude for your erudite advice, commitment, and encouragement to both my project and to my development as a student. Thank you for taking the time to help develop me as a young scientist and sharing your pearls of wisdom.

Last, but most certainly not least, I must thank my supervisor, Dr. Naranjan S. Dhalla, for taking a risk in having me as the final student in his laboratory. You were a constant

source of advice and wisdom. Your steadfast commitment to unravelling the mysteries of cardiovascular disease was a beacon of inspiration and I am most grateful that I had the chance to learn from such an exceptional mentor. Thank you, Dr. Naranjan S. Dhalla, for being such a wonderful supervisor who consistently kept my mind busy learning about the subtle intricateness of subcellular remodelling in cardiovascular disease.

ABSTRACT

Previous studies have shown that ischemia-reperfusion (I/R) injury is associated with cardiac dysfunction and depression in sarcolemmal Na^+-K^+-ATPase activity. This study was undertaken to evaluate the role of proteases in these alterations by subjecting rat hearts to different times of global ischemia, and reperfusion after 45 min of ischemia. Decreases in Na^+-K^+-ATPase activity at 60 min of global ischemia were associated with augmented activities of both calpain and MMPs and depressed protein content of β1- and β2-subunits, without changes in α1- and α2-subunits of the enzyme. However, reperfusion of ischemic heart produced depression in Na^+-K^+-ATPase activity, no change in the augmented calpain activity, but decreases in augmented MMP-2 activity and Na^+-K^+-ATPase content. MDL28170, a calpain inhibitor, was more effective in attenuating I/R-induced alterations than doxycycline, an MMP inhibitor. Incubation of control SL preparation with calpain, unlike MMP-2, depressed Na^+-K^+-ATPase activity and decreased α1, α2 and β2 without changes in β1. These results support the view that activation of calpain is involved in depressing Na^+-K^+-ATPase activity and degradation of its subunits in hearts subjected to I/R injury.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

ABSTRACT iii

TABLE OF CONTENTS iv

LIST OF ABBREVIATIONS vii

LIST OF FIGURES ix

LIST OF TABLES x

I. REVIEW OF LITERATURE 1

1. Pathophysiology of Ischemia-Reperfusion Injury 2

2. Na^+-K^+-ATPase 4

3. Extracellular and intracellular proteases 4

4. Calpain in ischemia-reperfusion injury 8

5. Matrix metalloproteinases in ischemia-reperfusion injury 12

6. Lysosomal cathepsins in ischemia-reperfusion injury 16

7. Peptidases, serine proteases, and caspases in ischemia-reperfusion injury . . . 18

8. Subcellular remodelling due to proteolytic activity 22

II. STATEMENT OF PROBLEM AND HYPOTHESIS 24

1. Ischemia-induced changes in cardiac function, Na^+-K^+-ATPase activity and subcellular degradation, and protease activation 25

2. I/R-induced changes in cardiac function, Na^+-K^+-ATPase activity and subcellular degradation, and protease activation 26

3. Direct effect of proteases on Na^+-K^+-ATPase activity and subcellular degradation 26

3. Hypothesis 27

III. MATERIALS AND METHODS 28

1. Perfusion and experimental protocol 28

2. Isolation of SL membrane 29

3. Incubation of SL with active proteases 30

4. Measurement of SL Na^+-K^+-ATPase activity 30

5. Western blot analysis 30

6. Measurement of calpain activity 31

7. Measurement of MMP-2 activity 32

8. Statistical Analysis 32

IV. RESULTS 33

1. Cardiac Dysfunction in Ischemic and I/R Hearts 33

2. Time-course alterations of protease and Na^+-K^+-ATPase activities and sub-unit degradation 35

3. Effect of perfusion with calpain and MMP-2 inhibitors on protease activities and Na^+-K^+-ATPase activities and subunit concentrations 39

4. Effects of active calpain and MMP-2 on Na^+-K^+-ATPase activity and subunits in isolated SL membrane 42

V. DISCUSSION 44

1. Cardiac dysfunction in ischemia and ischemia-reperfusion 44

2. Depression in Na^+-K^+-ATPase activity due to global ischemia and reperfusion 45

3. Alterations in Na^+-K^+-ATPase subunits due to global ischemia and reperfusion 46

4. Activation of calpain and MMP-2 due to global ischemia and reperfusion . . .47

5. Effects of protease inhibition in ischemic and reperfused hearts 48

VI. FUTURE DIRECTIONS 51

VII. CONCLUSIONS 52

VIII. REFERENCES 54

List of Abbreviations

ACE – angiotensin converting enzyme

APP – aminopeptidase P

Dox - doxycycline

DPP-4 – dipeptidyl peptidase-4

ECM – extracellular matrix

GSK-3β – glycogen synthase kinase 3-β

HtrA2 – high temperature requirement A2

I/R – ischemia-reperfusion injury

KO – knock-out

LTCC – L-type Ca^{2+} channels

LV – left ventricle

LVDP – left ventricular developed pressure

LVEDP – left ventricular end-diastolic pressure

MDL – MDL28170

MI – myocardial infarction

MLC-1 – myosin light chain-1

MMP – matrix metalloproteinases

Mn-SOD – manganese superoxide dismutase

MT1-MMP – membrane-type matrix metalloproteinases

NCX – Na^{+}-Ca^{2+} exchanger

NO – nitric oxide

NOS – nitric oxide synthase

ROS – reactive oxygen species

RyR2 – ryanodine receptor-2

SERCA – sarcoplasmic reticulum Ca^{2+}-stimulated ATPase

SL - sarcolemma

SR – sarcoplasmic reticulum

TIMP – tissue inhibitor of matrix metalloproteinase

List of Figures

Figure 1. SL Na^+-K^+-ATPase, as well as cytosolic calpain, and MMP-2 activities from hearts collected at 15, 30, and 60 minutes of ischemia and hearts reperfused at 0, 5, 10, 20 and 40 minutes after 45 minutes of ischemia 36

Figure 2. Protein content of Na^+-K^+-ATPase α-1 and α-2 subunits in SL preparations from hearts collected at 15, 30, and 60 minutes of ischemia and hearts reperfused at 0, 5, 10, 20 and 40 minutes after 45 minutes of ischemia 37

Figure 3. Protein content of Na^+-K^+-ATPase β-1 and β-2 subunits in SL preparations from hearts collected at 15, 30, and 60 minutes of ischemia and hearts reperfused at 0, 5, 10, 20 and 40 minutes after 45 minutes of ischemia 38

Figure 4. SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as cytosolic calpain, and MMP-2 activities of the heart subjected to 30 min ischemia and 30 min reperfusion in the absence or presence of calpain inhibitor, MDL28170 (MDL), or MMP-2 inhibitor, doxycycline (Dox) 40

Figure 5. Protein content of Na^+-K^+-ATPase subunits in SL preparations from hearts subject to ischemic reperfusion (I/R) injury perfused in the absence or presence of calpain inhibitor, MDL28170 (MDL), or MMP-2 inhibitor, doxycycline (Dox) 41

Figure 6. Na^+-K^+-ATPase activity, Mg^{2+}-ATPase activity, and Na^+-K^+-ATPase subunit protein content in isolated LV SL treated with active calpain and/or active MMP-2 43

List of Tables

Table 1. Alterations in cardiac function (LVDP and LVEDP) in hearts subjected to global ischemia for different times and reperfusion of the 45 min ischemic heart for different times, as well as ischemia reperfusion injury in hearts perfused in the absence or presence of protease inhibitors 34

I. REVIEW OF LITERATURE

The pathophysiology of cardiac dysfunction in heart disease is a complex process that involves the interplay and alterations of various extracellular and intracellular proteins and molecules. One of the key families of enzymes involved is the proteases which specialize in cleaving protein peptide bonds (Singh et al. 2004b). Various studies in the area of heart disease have shown their involvement in cardiac remodelling, a phenomenon that occurs partly as a result of ischemia–reperfusion (I/R) injury (Singh et al. 2004b). This injury arises from cessation of nutrients perfusing the heart due to lack of blood flow and is associated with initial damage that becomes significantly amplified upon reperfusion. Bursts of reactive oxygen species (ROS) occurring upon reperfusion lead to oxidative stress and the development of intracellular Ca^{2+}-overload, thus impairing a complete recovery of cardiac function (Dhalla et al. 2000; Dhalla et al. 2007; Ytrehus et al. 1987). The cessation and subsequent reperfusion of blood flow occurs in a number of clinical procedures including angioplasty, thrombolytic therapy, coronary bypass surgery, and cardiac transplantation (Dhalla et al. 2000). The detrimental action of I/R injury occurs during the requisite reperfusion period, where oxidative stress induced by ROS and increases in intracellular Ca^{2+} can catalyse the activation and modification of numerous proteins in the cell, plausibly altering their functions (Dhalla et al. 2000). Thus, the disequilibrium of Ca^{2+} homeostasis and generation of ROS have the potential to directly and/or indirectly activate different proteases in the heart to promote the development of cardiac dysfunction following I/R injury.

Proteases are essential for the homeostatic maintenance of the cell, allowing for the degradation of misfolded or malfunctioning proteins, and routine turnover of the

extracellular matrix (ECM) and other subcellular organelles (Cuervo et al. 2010; Rodriguez et al. 2010; Willis et al. 2009). Proteases are active at a basal level in cardiomyocytes. However, their actions are controlled via regulation of their transcription, translation, chaperone molecules, and endogenous inhibitors. On the other hand, under pathological conditions including I/R injury, these regulatory and control mechanisms are altered leading to marked increases in protease activities (Chohan et al. 2006; Cuervo et al. 2010; Singh et al. 2004a; Singh et al. 2004b; Powell et al. 2005; Temsah et al. 1999; Willis et al. 2009). These changes can occur both intracellularly and extracellularly, depending on the type of protease and location of its activation, as well as on interactions with its target. The proteases present in the myocardium that have demonstrated involvement in I/R injury include calpain, matrix metalloproteinases (MMPs), and cathepsins. The details regarding the structure, localization and methods of activation of calpains, MMPs, and cathepsins can be found in extensive reviews (Ali and Schulz 2009; Dhalla et al. 2000; Dhalla et al. 2007; Huang and Wang 2001; Kar et al. 2010; Mort and Buttle 1997; Perrin and Huttenlocher 2002; Reiser et al. 2010; Rodriguez et al. 2010; Singh et al. 2004b; Spinale 2001; Spinale 2007; Turk et al. 1997; Wilson and McDonald 1985; Zaidi et al. 2008).

1. Pathophysiology of Ischemia-Reperfusion Injury

Ischemia-reperfusion injury occurs when blood perfusion to the heart ceases, depriving it of essential nutrients such as oxygen and glucose, and then blood flow is re-introduced upon reperfusion (Singh et al. 2004b). This phenomenon occurs in numerous clinical procedures such as thrombolytic therapy, coronary bypass surgery, angioplasty, and cardiac transplantation (Müller et al 2012). It has been observed that the majority of

damaged caused in I/R injury is a result of oxidative stress and the development of intracellular Ca^{2+}-overload which trigger cardiac remodelling and prevent complete cardiac recovery from occurring (Dhalla et al. 2007; Singh et al. 2004b; Ytrehus et al. 1987). It has been shown that I/R induced intracellular Ca^{2+}-overload can further exacerbate ROS production resulting in increased oxidative stress (Dhalla et al. 1999). The subcellular organelle responsible for the majority of ROS production is the mitochondria, where cytochromes, xanthine oxidoreductase, NAD(P)H oxidase, and nitric oxide synthase (NOS) reside (Penna et al. 2009). It has been observed that in ischemia the mitochondria are in a reducing state; however, upon reperfusion with the introduction of oxygen, various investigations have observed increases in ROS production (Dhalla et al. 2000; Vanden Hoek et al. 1998). In addition to ROS being released from the mitochondria, the shift in states from aerobic to anaerobic metabolism leads to acidosis of the heart and can activate Ca^{2+}-dependent enzymes (Zaugg and Schuab 2003). Specifically, the sarcoplasmic reticulum (SR), has been shown to have modified Ca^{2+} cycling activities in I/R injury has a result of decreases in sarcoplasmic reticulum Ca^{2+}-stimulated ATPase (SERCA) activity and increasing Na^{+}-Ca^{2+} exchanger (NCX) activity (Kuster et al. 2010). The sarcolemma has also shown to be physically disrupted by oxyradicals resulting in intracellular exposure to the extracellular environment (Arora and Hess 1985). I/R injury has many negative influences on cardiac homeostasis as a result of nutrient depravation including altering Na^{+}-K^{+}-ATPase activity (Singh et al. 2012).

2. Na^+-K^+-ATPase

Maintaining ionic homeostasis in cardiomyocytes is essential for optimal cardiac function. Na^+-K^+-ATPase is an ion pump responsible for maintaining the ideal concentrations of Na^+ and K^+ ions inside and outside the cell. There are numerous isoforms present in both the adult rodent and adult human hearts. The predominant isoform is Na^+-K^+-ATPase α-1 (62% in humans) which is associated with the Na^+-K^+-ATPase β-1 subunit (Zahler et al. 1993) whereas Na^+-K^+-ATPase α-2 is associated with the Na^+-K^+-ATPase β-2 subunit. There are also differences in the distribution of the Na^+-K^+-ATPase isoforms. Na^+-K^+-ATPase α-1 is ubiquitously present throughout the heart whereas the α-2 isoform is specifically concentrated near the sarcoplasmic reticulum where it may indirectly influence the Na^+-Ca^{2+}-exchanger as well as nearby the t-tubules (Juhaszova and Blaustein 1997; Swift F et al. 2007; Berry et al. 2007). It has also been shown recently in mice that Na^+-K^+-ATPase α-2 has more influence on modulating Ca^{2+} release of cardiomyocytes (Despa et al. 2012). Although studies have been performed that look at changes in Na^+-K^+-ATPase activity in I/R injury (Singh et al. 2012), there is very little work that specifically investigates the role of its various subunits and isoforms which could be an important key in understanding cardiac malfunction in I/R injury. In addition, discovering at which point during I/R injury a crucial Na^+-K^+-ATPase unit is malfunctioning or being degraded could unveil more about what genuinely causes I/R injury and when and where cardiac dysfunction originates.

3. Extracellular and intracellular proteases

Proteolysis of both intracellular and extracellular proteins is important as the homeostasis of the intracellular milieu in conjunction with the stability of the surrounding cardiac interstitium is crucial in normal heart function. The digression from normal cardiac architecture by proteolytic degradation leads to abnormal cardiac function and cellular damage (Brilla and Maisch 1994). Traditionally perceived as being a relatively static cellular scaffold, it has become clear that the cardiac extracellular environment is as important in the function of the heart as the intracellular milieu of the cardiomyocyte. The ECM environment contains signalling molecules, proteases, cytokines and growth factors that appear to be compartmentalized throughout the interstitium (Baumgarten et al. 2002; Dell'Italia et al. 1997; Diwan et al. 2004; Ergul et al. 2000; Lee and McCulloch 1997; Miner and Miller 2006; Sivasubramanian et al. 2001; Spinale 2007). Regardless of the bioactive molecules present throughout the cardiac interstitial space, the architecture of matrix proteins themselves is distinctively arranged to optimize communication between cardiomyocytes and the overall pumping action of the heart (Streeter and Bassett 1966; Streeter et al. 1969). During I/R injury, ECM is degraded via proteolysis to enable infiltration of fibroblasts to the damaged area for commencing the process of wound repair (Spinale 2010); however, such a change in ECM, as well as in cardiomyocytes, may also result in cardiac dysfunction. Extracellular integrity is as important to maintain as cellular integrity, and thus alteration of the ECM by proteases is of critical importance to consider when targeting proteolytic activity.

The majority of extracellular damage is caused by the MMP family which degrades a number of ECM structural proteins including collagen, fibronectin, elastin,

and proteoglycans (Hobeika et al. 2008). Vanhoutte et al. (2006) have analysed the temporal alterations in the ECM with respect to MMP activity and have suggested that damage of the ECM is dependent on the duration of ischemic insult. An imbalance between MMPs and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), has been noted indicating that the potential inhibitory effect of TIMPs is overpowered by the accentuation of MMP activity (Lalu et al. 2005; Schulz 2007; Schulze et al. 2003). MMPs have also recently been attributed to causing proteolysis of various sarcomeric structural proteins (Ali et al. 2010; Wang et al. 2002; Sung et al. 2007; Sawicki et al. 2005).

Changes in the intracellular milieu, such as an increase in the concentration of Ca^{2+}, activate calpain causing it to degrade numerous subcellular organelle proteins. In addition, this increase in calpain activity is partially caused by the degradation of its endogenous inhibitor, calpastatin (Golstein and Kroemer 2007; Huang and Wang 2001; Kar et al. 2010; Ma et al. 2001; Perrin and Huttenlocher 2002; Samanta et al. 2010a; Singh et al. 2004a). It is also important to note that ECM damage can occur via cathepsins by the proteolytic cleavage of collagen and other interstitial structural proteins (Cheng et al. 2006; Everts V et al. 2006; Felbor et al. 2000; Helske et al. 2006; Jane-Lise et al. 2000; Maciewicz and Etherington 1988; Schenke-Layland et al. 2009; Yasuda et al. 2004). Lysosomal changes occur within the ischemic cardiomyocyte and have been observed during prolonged I/R and alterations in cathepsins occur during peak ischemic injury (Decker and Wildenthal 1980; Ganote et al. 1975; Hearse et al. 1973; McCallister et al. 1977). Although the activities of the above mentioned proteases are modified by changes in pH, it is not clear whether the activities of endogenous inhibitors

of these proteases are altered by pH as well. Overall, it is reasonable to suggest that the extent of alterations in the heart, both to the ECM and cardiomyocytes, by I/R injury is influenced by the vigour of proteolytic activities in the myocardium.

It should be noted that the decrease in pH during ischemia occurs as a result of anaerobic by-products, including lactate and hydrogen ions despite the presence of the Na^+/H^+ exchanger (Cardone et al. 2005; Inserte et al. 2009; Neely and Grotyohann 1984). Low pH has been demonstrated to affect both ECM remodeling and intracellular protease activities as protease classes are sensitive to various pH levels (Cardon et al. 2005; Inserte et al. 2009; Powell and Matrisian 1996). Acidic proteases, particularly those belonging to the family of cysteine proteases and aspartyl cathepsins, have been reported to increase their proteolytic activities under acidic conditions which further exacerbate ECM remodelling (Cardone et al. 2005). Cathepsins are effective at acidic pH values where low extracellular pH (pHe) has been demonstrated to increase the release of cathepsin B (Cardone et al. 2005). In cells cultured in an acidic environment, there was increased secretion of active cathepsin B (Webb et al. 2001). With respect to the MMP family, although some isoforms have been observed to have reduced proteolytic activities at low pH, not all MMPs require proteolytic cleavage in their conversion from their zymogen form to an active form (Martinez-Zaguilan et al. 1996). In fact, a member of the gelatinase MMP family has shown to be activated by acid treatment (Davis and Martin 1990). Even at an acidic pH of 6.8, MMPs have been observed to retain up to 80% of their proteolytic activities (Turner 1979). A significant amount of acidosis has been noted to increase caspase-3 activity, which propagates the apoptotic cascade in cardiomyocytes (Frazier et al. 2006). However, it has also been observed that prolonging

acidosis via preconditioning inside the cell can cause a cardioprotective effect, particularly concerning the attenuation of calpain proteolytic damage (Hernando et al. 2010; Inserte et al. 2009). Since pH is known to decrease in the ischemic myocardium and to show dramatic alterations upon reperfusion, it appears that the activities of different proteases are modified by changes in pH during the development of I/R injury. It is noteworthy that there is very little information regarding the influence of changes in pH on the behaviour of endogenous inhibitors of proteases available in the literature.

4. Calpain in ischemia–reperfusion injury

Calpain is a Ca^{2+}-dependent cysteine protease involved in the degradation of various structural proteins and the cell death pathway. Its involvement in contributing to cardiovascular damage in I/R injury is demonstrated by its inhibition which results in a reduced infarct size (Iwamoto et al. 1999; Mani et al. 2009). However, it appears to only become activated post-reperfusion once cellular pHi has stabilized because when pHi is lowered, the decrease in calpain activity is comparable to when it is inhibited by a pharmacological calpain-specific inhibitor, MDL-28170 (Inserte et al. 2009). Calpain targets troponin I (TnI), a component of the actin–tropomyosin complex, which has been noted to be degraded during I/R injury (Schwartz et al. 2003). Titin, the elastic filament in sarcomeres that has been proposed to provide a scaffold for the assembly of other myofilaments (Granzier and Labeit 2004), has also been found to be a target of calpain activation, although a 24 h incubation time with cardiotoxic doxorubicin was required in this report (Lim et al. 2004). Another substrate of calpain is α-fodrin, a key component of the cytoskeleton that forms a three-dimensional mesh-like network in cardiac

myocytes (Kobayashi et al. 2004; Larsen et al. 1999; Tsuji et al. 2001; Yoshikawa et al. 2005). It has been implied that, due to the linking function of α-fodrin in the cytoskeleton, proteolysis of α-fodrin may alter the properties of ion channels (Larsen et al. 1999). For example, L-type Ca^{2+} channels (LTCC) have been observed to exhibit decreased activities upon disturbance of cytoskeletal proteins (Galli and DeFelice 1994; Yoshida et al. 1995). A novel calpain inhibitor, SNJ-1945, counteracted α-fodrin degradation without affecting LTCC protein levels indicating that the likelihood that α-fodrin may be a tethering anchor between LTCC and the membrane, possibly playing a role in regulating the basal activity of the channel. The proteolysis of α-fodrin by calpain impairs its function whereas calpain inhibition by SNJ-1945 preserves its connection to the membrane (Galli and DeFelice 1994; Nakamura et al. 2000; Yoshikawa et al. 2010). However, a study done by Gilchrist et al. (2010) evaluating calpain degradation of various components in the cell as a result of global no flow I/R model using Sprague–Dawley rat hearts, found that α-fodrin degraded in a manner uncharacteristic of calpain. Thus, it was proposed that calpain could be activated by other proteases such as trypsin, chymotrypsin, and caspase-3 which are also known to degrade α-fodrin (Gilchrist et al. 2010; Harris and Morrow 1998; Nath et al. 1996). This is supported by the lack of activated calpain subunits found in the cytosolic fractions of the heart as determined by immunostaining technique (Gilchrist et al. 2010). In addition to proteolysis of α-fodrin, calpain-I has been shown to degrade desmin in post-ischemic myocardium, reducing maximal isometric force and contributing to sarcomere disorder (Papp et al. 2000). Cleavage of cytoskeletal proteins would impair the contractile unit of the cardiomyocyte

resulting in a decreased ability to contract effectively, contributing to overall cardiac dysfunction.

The proteolytic cleavage resulting in reduced levels of intact sarcoplasmic reticulum (SR) Ca^{2+}-cycling proteins has also been suggested to occur via calpain action (Singh et al. 2004a). In addition, degradation of 50–70% of basal ryanodine receptor-2 (RyR2) tissue content has been noted in ischemia and after I/R (Domenech et al. 2003; Temsah et al. 1999). Data have been presented suggesting that calpain is responsible for the degradation of RyR2 as protein content of RyR2 was reduced by half, however mRNA levels did not change (Pedrozo et al. 2010). Upon administration of leupeptin, a cysteine protease inhibitor known to act on calpain, RyR2 protein damage was attenuated which yielded similar information when compared to results where calpain was inhibited by E64d, a specific calpain inhibitor (Gilchrist et al. 2010). However, it has also been found that RyR2 fragmentation occurs in I/R injury, but its degradation may not be effected by calpain (Harris and Morrow 1988); Gilchrist et al. (2010) also observed that SERCA fragmentation coincided with calpain cleavage. Accordingly, it was suggested that although SERCA is not recognized as a calpain substrate *in vitro*, its potential alterations by oxidative stress could facilitate its cleavage by calpain (Singh et al. 2004a; Temsah et al. 1999). It is pointed out that the sarcolemma has been found to be susceptible to calpain activation as the autolytic portion of calpain in hearts that underwent I/R injury was found to be in excess of that in control hearts (Gilchrist et al. 2010). These changes to RyR2 and SERCA influence Ca^{2+} homeostasis by impairing SR Ca^{2+} uptake which may contribute to Ca^{2+}-overload (Temsah et al. 1999).

In a study examining the relationship between I/R and H_2O_2 induced calpain activation and estrogen, it was found that estrogen played a role in cardiac protection as it inhibited the calpain-mediated signaling streams. This was shown by the finding that hearts of ovariectomized rats had higher levels of calpain activity, where treatment of these mice with estrogen significantly decreased I/R-induced calpain activation (Chae et al. 2007). Another mechanism observed to decrease calpain activation was found in exercising animals. In these animals, reductions have been noted in I/R-induced pro-apoptotic proteins, which are comparable to observations in hearts treated with pharmacological calpain inhibitors MDL-28170 and calpain inhibitor 3 (French et al. 2008; French et al. 2006). The mechanism proposed is that exercise causes an increase in myocardial manganese superoxide dismutase (Mn-SOD) which prevents I/R-induced modification via oxidative stress of Ca^{2+}-handling proteins, thus reducing calpain activation (French et al. 2008). Calpastatin, the endogenous inhibitor of calpain present in both 70 and 110 kDa forms, has also been observed to be decreased in I/R injury, therefore allowing calpain to cause destruction within the cell with minimal resistance (Samanta et al. 2010b; Singh et al. 2004a). Ischemia for 20 min followed by 30 min of reperfusion causes a decrease in calpastatin activity. After comparison of the molecular fragment masses between proteolysis of calpastatin in vitro with superfluous calpain and the proteolysis in the I/R heart, it was noted that the fragment masses were significantly similar suggesting that the loss of calpastatin may be due to its proteolysis by increased quantities of active calpain (Sorimachi et al. 1997). Due to its sensitivity to increased intracellular levels of Ca^{2+}, calpain is definitely an intracellular protease that

should be taken into consideration while evaluating proteolytic damage caused by I/R injury.

5. Matrix metalloproteinases in ischemia–reperfusion injury

Although a significant amount of literature attributes damage induced by I/R injury to calpain activity, it has been shown that some inhibitors of calpain, in particular calpastatin, can inhibit an entirely different family of proteases, the MMPs, specifically MMP-2 (Kandasamy et al. 2010). MMPs are endopeptidases that utilize a highly-conserved zinc-binding catalytic center in order to cleave proteins, originally discovered to be instrumental in tadpole morphogenesis (Ali and Schulz 2009; Gross and Lapiere 1962). MMP-2 has been demonstrated to contribute significantly to I/R injury (Cheung et al. 2000; Fert-Bober et al. 2008; Lalu et al. 2005; Wang et al. 2002). Furthermore, increased MMP-2 activity has been shown in H9c2 cardiomyocytes exposed to oxidative stress, along with glycogen synthase kinase 3-β (GSK-3β) cleavage and increased GSK-3β activity; all these changes were attenuated with MMP inhibitors (Kandasamy et al 2009). In addition, MMP-9, another gelatinase, has also been noted to be expressed during I/R injury (Tiwari et al. 2008).

The classic view of MMPs restricts their proteolytic activities to the ECM where they were shown to be responsible for partial degradation of the interstitial matrix (Ali and Schulz 2009) as well as cell adhesion proteins (Rodriguez et al. 2010). Elevation of both MMP-2 and MMP-9 has been shown in plasma post MI in human hearts within the first minute of reperfusion (Lalu et al. 2005). Markers of ischemic damage, particularly creatine kinase, serrotransferrin, interstitial albumin, and overall cardiac edema were

diminished upon inhibition of MMP-2 using *o*-phenanthroline or doxycycline (Fert-Bober et al. 2008). The significance of MMP-2 activity in accentuating damage to the heart has been reported by studying constitutively active MMP-2 transgenic mice, which show decreases in functional recovery after I/R injury (Zhou et al. 2007). The extent of ECM damage caused by ischemia could be correlated directly with the activation of MMP-2 and inversely to the recovery of mechanical function in human atrial muscle (Lalu et al. 2005). Interestingly, a study evaluating collagen ultrastructure post I/R injury *in vivo* found that, although in moderate ischemia there was an increase in MMP-2 and MMP-9 content and activity, the degradation of the interstitial collagen was minimal (Lu et al. 2000). It has also been observed that extracellular MMP-9 has an effect on intracellular calcium which is mediated via the proteinase-activated receptor-1 (PAR-1), a transmembrane G-protein-coupled receptor (Macfarlane et al. 2001; Mishra et al. 2010). PAR-1 antagonism and MMP-9 knockout (KO) each improved cell shortening and increased the relaxation velocity of cardiomyocytes. It was found that in MMP-9 KO cells, SERCA2a is upregulated, which may contribute to the increased contractility of the cardiomyocyte (Mishra et al. 2010). This demonstrates the potential of extracellular MMPs to influence changes within the cardiomyocyte that may contribute to the damage induced by I/R injury.

Not only can extracellular MMPs directly influence what is occurring outside the cardiomyocyte, recent work has shown the presence of MMP activity intracellularly. Although MMP-2 has been localized inside various cellular organelles including the nucleus and mitochondria (Kandasamy et al. 2010), it was reported to migrate to the sarcomere. MMP-2 co-localizes with troponin I (TnI) in the thick and thin myofilaments

and proteolytically cleaves them, diminishing intracellular integrity (Wang et al. 2002; Sawicki et al. 2005). It has also been shown to cleave α-actinin and myosin light chain-1 (MLC-1) impairing sarcomeric function (Sawicki et al. 2005; Sung et al. 2007). Studies in isolated rat cardiomyocytes exposed to simulated ischemia have revealed that MLC-1 is nitrated/nitrosylated via $ONOO^-$ (peroxynitrite) exposure at specific Tyr residues. This resulted in its increased degradation by MMP-2, which corresponded with *in vitro* MMP-2 degradation of human recombinant MLC-1 after $ONOO^-$ exposure; this change was attenuated when an $ONOO^-$ scavenger, FeTPPS, was added (Polewicz et al. 2011). It appears that, not only is MMP-2 activated by oxidative stress but one of its substrates also has increased susceptibility to proteolytic cleavage, therefore impairing cardiomyocyte infrastructure and further amplifying the damage caused by ROS. An additional sarcomeric protein has been added to the roster of those degraded by MMP-2 and that is the molecular giant titin (Ali et al. 2010). One of its major functions is to provide elasticity to the sarcomere and its degradation significantly compromises the contractility of the sarcomere, which has been observed in ischemic hearts (Tskhovrebova and Trinick 2010). In this regard, purified titin as well as titin in skinned cardiomyocytes was degraded in a concentration-dependent manner when incubated with MMP-2. Isolated rat hearts subjected to I/R injury revealed titin fragmentation which was attenuated with MMP-2 inhibition using ONOO′-4817. Furthermore, co-localization between titin and MMP-2 was observed on the Z-disk region of titin using confocal fluorescent microscopy in human cardiomyocytes. Finally, in MMP-2 KO mice, when coronary artery ligation was applied for 30 min, followed by 30 min of reperfusion, there was significantly less titin degradation than in wild-type

control hearts (Ali et al. 2010). The proteolytic cleavage of cardiomyocyte structural proteins by MMP-2 also contributes to impairing overall cardiac function by disabling the crucial contractile components of the sarcomere. It is thus evident that the intracellular involvement of MMP-2 is a critical factor in determining the extent of damage suffered by the heart due to I/R injury.

The endogenous inhibitors of MMPs are TIMPs 1–4, which are found throughout the body including the heart (Baker et al. 2002). Ischemic post-conditioning has been demonstrated to be partially effective in inhibiting MMP-2 activity; however, the precise mechanism has yet to be elucidated (Donato et al. 2010). Disequilibrium has been noted between TIMPs and MMPs during I/R injury which may contribute to the additional damage occurring to the heart (Lalu et al. 2005; Schulz 2007; Schulze et al. 2003). In particular, TIMP-4, the most abundant TIMP expressed in the heart, decreases in the coronary effluent in relation to increased durations of ischemia (Schulze et al. 2003). Interestingly, during coronary artery bypass surgery, atrial biopsies indicated a decrease in TIMP-1 but not TIMP-4 (Lalu et al. 2005). When mRNA levels were compared between MMPs and TIMPs in a rat model of MI, MMP mRNA was increased; however, a concomitant increase in TIMP mRNA did not occur (Peterson et al. 2000). TIMP-4 has also demonstrated susceptibility to $ONOO^-$ modification via the nitration of crucial tyrosine residues and oligomerization rendering it ineffective against MMPs (Donnini et al. 2008). In addition to MMPs, there are membrane-type MMPs (MT-MMP), which are cell surface activators of MMPs that are physiologically attached to the cell membrane (Massova et al. 1998). Specifically MT1-MMP has been demonstrated to have increased interstitial activity upon induction of I/R (Deschamps et al. 2008; Deschamps et

al. 2005). In addition, MT1-MMP deficient mice demonstrated inadequate collagen turnover and died within the third week of life (Holmbeck et al. 1999; Holmbeck et al. 2004). The activation of MMP-2 is mediated by MT1-MMP via the formation of a complex between MT1-MMP and TIMP-2, which indicates that, not only is TIMP-2 responsible for MMP-2 inhibition, under certain circumstances TIMP-2 is involved in MMP-2 activation (Strongin et al. 1995). MMPs, TIMPs, and MT-MMPs are intricately involved with both extracellular and intracellular modifications of cardiac proteins during I/R injury.

6. Lysosomal cathepsins in ischemia-reperfusion injury

Although calpain and MMPs are the major culprits of proteolytic damage occurring in I/R injury, it is important to consider an additional family of proteases, the cathepsins, which are the primary protease family found in the acidic environment of lysosomes (Tiwari et al. 2008; Turski and Zaslonka 2000). Changes of lysosomes in I/R injury have been observed when studies examined how hypoxia causes lysosomes to alter their phenotype based on the duration of hypoxia exposure (Decker and Wildenthal 1980). At 40 min of hypoxia, lysosomal activation appears to increase and potentially correlates with mitochondrial degradation (Decker and Wildenthal 1980; McCallister et al. 1977). At 60 min of hypoxia, lysosomes coalesce with each other and additional membranes, and irreversible injury occurs (Ganote et al. 1975; Hearse et al. 1973). Subsequently, upon reperfusion, there is sustained cardiac dilation and minimal contractile activity (Decker and Wildenthal 1980). This study concluded that, in cells sustaining minimal damage, lysosomes appear to function normally by digesting

organelles no longer functioning properly; however, in lethally injured myocytes, upon reperfusion, the enlarged lysosomes do not appear to remove the intracellular debris and thus ultimately prevent proper cellular repair (Baker et al. 2002).

In cardiomyocytes, the lysosomal cysteine proteases, cathepsins, have been shown to remain inactive for short periods of ischemia; however, ischemic periods extending over an hour can trigger their activation (Kitakaze et al. 1988; Matsumura et al. 1993; Wildenthal 1978). Cathepsins D and L are of keen interest during I/R injury as they are partially responsible for myofibrillar protein degradation (Sorimachi et al. 1997). Cathepsin D, in particular, has been shown to have increased activity in correlation with increased duration of I/R in rat hearts (Decker et al. 1997; Tiwari et al. 2008; Turski and Zaslonka 2000). These two cathepsins are currently the most studied in terms of their effects in I/R injury. Interestingly, results in patients undergoing coronary-artery bypass surgery (CABG) showed no change in the total and free activity of both cathepsins D and L in fragments of the right atrium. Nevertheless, it was found that during the CABG ischemic period, cathepsin D and L fragments were activated inside the lysosome but became inactive once they were released into the cytosol of the cell. During reperfusion, both intralysosomal and extralysosomal compartmental enzymes were inactivated (Turski and Zaslonka 2000). However, these studies failed to consider the extracellular activities of cathepsins, in particular cathepsin L.

Proenzyme cathepsin L can be secreted into the ECM and activated by MMPs resulting in the proteolytic cleavage of fibronectin, laminin, and type I, IV and XVIII collagen at neutral pH (Everts et al. 2006; Felbor et al. 2000; Maciewicz and Etherington 1988). Cathepsin L has also been shown to be important in neovascularization via

endothelial progenitor cells in degrading the ECM (Urbich et al. 2005). Neovascularization by endothelial progenitor cells has been shown to be important in re-establishing blood flow in ischemic areas (Chavakis et al. 2008; Jane-Lise et al 2000). Other cathepsins demonstrating ECM protein degradation capability include cathepsins B, K, S, and V (Cheng et al. 2006; Helske et al. 2006; Jane-Lise et al. 2000; Schenke-Layland et al. 2009; Yasuda et al. 2004). It should also be pointed out that the activity of cathepsin B has been implicated in the necrotic process of ischemic cardiomyocytes as it becomes activated when pHi becomes acidic (Wildenthal 1978). A reduction of lysosomal membrane integrity occurs due to lower adenosine triphosphate, pH and accumulation of membrane fatty acids during ischemia. This allows proteases to leak out and/or substrates to leak in resulting in abnormal breakdown of cellular components (Wildenthal 1978). In addition, the lowered pHi observed in ischemia is closer to the optimal pH of lysosomal enzyme activities which would potentiate degradation of proteins causing extensive damage and eventually apoptosis and/or necrosis (Wildenthal 1978). The leakage of lysosomal proteases could be a key factor in increased cardiomyocyte damage during I/R injury and warrants further investigation.

7. Peptidases, serine proteases and caspases in ischemia-reperfusion injury

Peptidases, in particular aminopeptidase P (APP), have shown involvement in I/R injury to the heart. APP is a zinc-specific metallopeptidase with two isoforms (Hooper et al. 1992); one interacts with the cellular membrane while the other is a cytosolic isoform (Yoshimoto et al. 1994). An emerging significant role of APP, speculated to be the membrane-bound isoform (Maggiora et al. 1999), is its kininase ability, where it has

been observed to be instrumental in metabolizing bradykinin, an agent involved in regulating blood pressure and cardiac function (Liu et al. 1996; Miura 1996; Naitoh et al. 1997; Orawski et al. 1989; Prechel et al. 1995; Remme 1997; Scholkens 1996; Siragy 1993). It acts by removing the N-terminal amino acid and cleaving the bond between Arg^1 and Pro^2 (Simmons and Orawski 1992). Bradykinin releases inositol triphosphate (IP_3) and diacylglycerol (DAG) via phospholipase C and activates protein kinase C. These products have been shown to be beneficial in reducing arrhythmias and improving contractility (Rett et al 1990). Bradykinin has been reported to reduce infarct size and decrease the duration of reperfusion arrhythmias (Martorana et al. 1990; Rajani et al. 1997; Wolfrum et al. 2001). Observations of increased myocardium protection have been made when endogenous bradykinin deactivation was inhibited causing the release of protective NO and prostaglandin (Massoudy et al. 1995; Schrör 1992; Shimada et al. 1996). It has been postulated to have the same level of involvement in myocardial kinin metabolism as angiotensin converting enzyme (ACE) (Dendorfer et al. 1997). APP inhibition by apstatin causes a decrease in both arrhythmias and the release of cytosolic enzymes in an *in vitro* model of ischemia (Ersahin et al. 1999). In a model of global ischemia, apstatin reduced ventricular fibrillation duration post 5-minute reperfusion (Ersahin et al. 1999). In fact, apstatin administration caused a similar reduction in myocardial infarct size as ACE inhibition (Akula et al. 2002; Annapurna et al. 2001; Veeravalli et al. 2003).

Another peptidase observed to have an effect in I/R is dipeptidyl peptidase-4 (DPP-4). Its primary functions include adenosine deaminase binding, peptidase activity, and ECM binding (Sauvé et al. 2010). It is known to cleave several proteins including

neuropeptide Y, and brain natriuretic peptide. In DPP-4 knockout mice and in wild-type diabetic mice treated with DPP-4 inhibitors sitagliptin or metformin, after undergoing experimental MI, an increased survival rate was observed (Sauvé et al. 2010).

Serine proteases have also been postulated to be involved in I/R injury to the heart. High temperature requirement A2 (HtrA2), a serine protease, translocates from the mitochondria to the cytosol of cardiomyocytes during I/R which promotes apoptosis via the caspase-mediated apoptotic pathway by degrading X-chromosome linked inhibitor of apoptosis protein (Bhuiyan and Fukunaga 2008). The inhibition of HtrA2 protease resulted in decreased infarct size in addition to attenuating decreases in post-ischemic myocardial contractile function (Bhuiyan and Fukunaga 2008). During CABG and myocardial infarction, high quantities of active thrombin have been measured (Schwertz et al. 2008) and the use of broad-spectrum serine protease inhibitors has also been found to provide whole organ protection during I/R for not only the heart, but also the kidney and liver (Horiuchi et al. 2001; Kher et al. 2005; Pruefer et al. 2002). For example, gabexate mesilate has demonstrated myocardial protective effects (Yamamoto et al. 1991) and the addition of nafamostat pre-reperfusion has resulted in significantly reduced myocardial injury (Horiuchi et al. 2001). Although there is some understanding on how I/R injury is affected by these families of serine proteases, it is evident that more studies are required to fully understand their potentially crucial roles in exacerbating I/R injury.

The degree of damage caused by I/R injury is also a factor in the degree of apoptosis that occurs within cardiomyocytes following this insult. Interestingly, the extent of apoptosis increases upon relief of ischemia by reperfusion (Gottlieb et al. 1994; Fliss and Gattinger 1996; Kang et al. 2000; Zhao et al. 2000). The magnitude of

cardiomyocyte apoptosis is indicative of the extent of I/R injury and is mediated, for the most part, by caspases. These caspases contribute by cleaving contractile proteins including actin, myosin, and troponin in addition to pro-apoptotic factors which cause the release of cytochrome *c* from mitochondria (Communal et al. 2002; Kostin et al. 2003). In addition, if DNA fragmentation fails to occur, caspase activation may not directly lead to apoptosis, especially as cytoplasmic protein degradation can occur which continuously contributes to substantially impairing contractile function (Kanoh et al. 1999; Narula et al. 1999; Narula et al. 1996). Different caspases have been found to have varying effects during I/R injury. In a study evaluating the differences between caspase-9, activated via mitochondrial damage initiating the intrinsic apoptotic pathway, and caspase-8, activated by Fas which mediates the extrinsic apoptotic pathway, there was an increase in caspase-9 cleavage in the endothelial cells of rat hearts exposed to ischemia or I/R. However, caspase-8 cleavage occurred exclusively in the cardiomyocytes during ischemia, which was enhanced upon reperfusion. Inhibition of caspase-9 in rat myocardium prevented I/R induced activation of the apoptotic cascade and decreased both infarct size as well as the release of lactate dehydrogenase and creatine phosphokinase (Sodhi et al. 2009). In addition to their pro-apoptotic activity, caspases have also been shown to activate MMPs, and their inhibition attenuated both regional and global LV remodelling in a porcine MI model (Yarbrough et al. 2010). Likewise, proteasomal inhibitors, Lac and MG 132, prevented the degradation of calpastatin as well as RyR in simulated I/R injury (Samanta et al. 2010b). When comparing the levels of 20S proteasome activity and ubiquitin between ischemic and non-ischemic regions of normal human hearts, activities were nearly undetectable (Marfella et al. 2009). This decline in proteasomal activity has been

noted to increase the quantity of mis-folded proteins in prolonged ischemia which may further exacerbate the injury and lead to currently unknown consequences (Dhalla et al. 2007; González et al. 2010). Additional investigation on how I/R affects the proteasome and its correlated proteins in the human heart is needed to fully understand the extent and mechanism of how apoptosis is triggered in order to potentially attenuate this phenomenon in the development of strategies for the treatment of patients who have suffered I/R injury.

8. Subcellular remodelling due to proteolytic activity

Various studies have evaluated the extent of subcellular remodelling that occurs in I/R injury, yet it is still to be established which proteases are responsible for certain remodelling targets (Dhalla et al. 2007). The sarcolemma (SL), SR, myofibrils, and mitochondria have all been shown to be victims of proteolytic degradation or alteration during I/R injury (Dhalla et al. 1988; Dhalla et al. 2000; Dhalla et al. 2007; Gao et al. 1997; Kusuoka et al. 1987; Ostadal et al. 2003; Ostadal et al. 2004; Ong and Hausenloy 2010; Singh et al. 2004a). This damage affects SL proteins, specifically the activity of the Na^+–K^+-ATPase which is depressed with decreases in α2, α3 and β1 subunit protein levels (Elmoselhi et al. 2003; Singh et al. 2008). In turn, this can have a negative impact on the Ca^{2+} efflux from the cardiomyocyte and may contribute to the I/R injury induced phenomenon of Ca^{2+}-overload, thus amplifying the proteolytic activities of calpain (Dixon et al. 1990; Golstein and Kroemer 2007; Perrin and Huttenlocher 2002). Changes have also been noted with regard to dystrophin degradation, phospholipase alteration, and membrane permeability changes (Asemu et al. 2003; Asemu et al. 2005;

Askenasy et al. 2001; Kyoi et al. 2003; Munakata et al. 2002). In addition, the SR sustains significant damage due to I/R, with notable impaired function of both Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+}-uptake in addition to decreased densities of Ca^{2+}-release channels (Kawabata et al. 2000; Osada et al. 1998; Temsah et al. 1999; Schoutsen et al. 1989; Yoshida et al. 1990; Zucchi et al. 1994; Zucchi et al. 2001). As previously mentioned, myofibrillar damage occurs during I/R, where MMP-2 appears to be a significant factor in this respect as it has been shown to cleave α-actinin (Sung et al. 2007), MLC-1 (Polewicz et al. 2011) and titin (Ali et al. 2010). In addition, troponin I and troponin T have also been observed to undergo proteolysis during I/R injury (Luciani et al. 1993; Makazan et al. 2007; Remppis et al. 1995; Westfall and Solaro 1992; Van Eyk et al. 1998). Similarly, mitochondria are affected by the stress caused by I/R injury and have been found to be significantly fragmented and have reduced state 3 respiration (Dhalla et al. 1988; Makazan et al. 2007). In the hearts of constitutively active MMP-2 transgenic mice, there were observed abnormal changes regarding mitochondrial morphology and respiration, lipid peroxidation, and diminished recovery (Lu et al. 2000). Although extensive work on remodelling of ECM and myofibrils due to the activation of protease activities has been carried out, it is noteworthy that relatively little information concerning remodelling of the SL, SR, and mitochondria is available in the literature. The mechanisms of I/R-induced subcellular remodelling have yet to be elucidated and the type of proteases responsible for the proteolytic degradation of each organelle needs to be clearly established.

II. STATEMENT OF PROBLEM AND HYPOTHESES

Although a number of reviews have been written describing the role of proteases in cardiovascular disease (Müller and Dhalla 2011; Müller et al. 2012a; Müller et al. 2012b; Singh et al. 2004b), there is much to learn regarding the effect of how proteolytic activation affects cardiovascular function in ischemia as well as in I/R. It has been suggested that various mechanisms, such as the development of oxidative stress and intracellular Ca^{2+}-overload, can contribute to cardiac dysfunction by increasing the proteolytic activities of proteases. Although earlier studies have shown that cardiac dysfunction due to I/R is associated with depressed activities of subcellular organelles, including SL, SR, myofibrils, and mitochondria, it is yet unknown at what stage of I/R does degradation of various subcellular organelles occurs and at what stage proteases may start affecting cardiac dysfunction. Therefore, in order to evaluate the sensitivity of Na^{+}-K^{+}-ATPase activity due to ischemia and I/R in this study, we evaluated Na^{+}-K^{+}-ATPase activity and its different subunit changes upon increasing the duration of ischemia in addition to altering reperfusion duration. It is important to note that the α-subunit is the catalytic portion of the enzyme whereas the β-subunit acts in a regulatory role by facilitating localization and assembly of the enzyme in to sarcolemma (Huang et al. 1994). Na^{+}-K^{+}-ATPase malfunction is associated with Ca^{2+} overload caused by the accumulation of Ca^{2+} ions by limiting Ca^{2+} extrusion via the Na^{+}/Ca^{2+} exchanger (Bers 2002; Bers 2008). Furthermore, the activities of calpain and MMP-2 were examined at these various stages to see at which point the proteolytic activities may influence degradation of sarcolemma. Inhibition of these proteases during I/R was also investigated to test its influence on cardiac function, Na^{+}-K^{+}-ATPase activity and sub-unit

degradation. To study whether calpain and MMP-2 exerts direct or indirect effects on Na^+-K^+-ATPase, the effects of both calpain and MMP-2 were examined on Na^+-K^+-ATPase activity and its subunit composition by incubating SL preparations with active forms of calpain and MMP-2. This investigation was undertaken to test the hypothesis that as damage induced by both ischemia and I/R, there is an increase in protease activity that correlates with a decrease in SL Na^+-K^+-ATPase activity, degradation of Na^+-K^+-ATPase sub-units that is different based on their sensitivity to degradation, and impaired cardiac function. The following three sets of experiments were carried out to gain some information on this regard.

1. Ischemia-induced changes in cardiac function, Na^+-K^+-ATPase activity and subcellular degradation, and protease activation

Ischemia is known to cause cardiac dysfunction, depress Na^+-K^+-ATPase activity and accumulate extracellular K^+, and as a result, an increase in intracellular Na^+ (Terkildsen et al. 2007). Experiments have shown that this disequilibrium of ions in addition to a reduction of available ATP for energy metabolism causes a reduction in Na^+-K^+-ATPase activity (Fiolet et al. 1984; Kleber 1983; Mitani and Shattock 1992). There are also changes in pH during ischemia brought on by an increase in lactate and hydrogen ions (Inserte et al. 2009; Neely and Grotyhann 1984) which have been shown to exacerbate proteolytic activity (Cardone et al. 2005). In addition MMPs can maintain up to 80% of their proteolytic activities at a pH of 6.8 (Turner 1979). When evaluating the role of proteases during ischemia, it was found that MMP-2 activity increases concomitantly with duration of ischemia (Cheung et al. 2000). These changes in the intracellular milieu contribute to the initial damage caused by ischemia; however, what is

currently unknown is the relative sensitivity of the Na^+-K^+-ATPase sub-units. We were also interested in evaluating the activity of both calpain and MMP-2 during the various stages of ischemia to see whether or not the anaerobic environment caused by ischemia would have an effect on protease activity and possibly contribute to the observed decrease of Na^+-K^+-ATPase activity.

2. I/R-induced changes in cardiac function, Na^+-K^+-ATPase activity and subcellular degradation, and protease activation

Although knowing what happens during ischemia is crucial in order to understand how damage to the myocardium may initially occur, reperfusion is necessary in order for the heart to be able to sustain itself and provide nutrients systemically. Unfortunately, the obligate re-entry of nutrients, particularly oxygen, puts additional stress and damage on the heart in the form of oxidative stress and intracellular Ca^{2+}-overload (Dhalla et al. 2000; Dhalla et al. 2007; Ytrehus et al. 1987). It has been established that significant damage is sustained by the heart in I/R injury as evident by impaired Na^+-K^+-ATPase activity and impaired cardiac function (Dhalla et al. 1988; Elmoselhi et al. 2003; Ostadal et al. 2003). As calpain isoforms are activated by various concentrations of Ca^{2+}, its activity has been shown to be elevated in I/R hearts (Chohan et al. 2006). We evaluated Na^+-K^+-ATPase activity and subunit degradation upon reperfusion. In addition, we investigated how inhibiting calpain and MMP-2 would affect Na^+-K^+-ATPase activity and subunit degradation.

3. Direct effect of proteases on Na^+-K^+-ATPase activity and subcellular degradation

In order to better understand how calpain and MMP-2 directly affect Na^+-K^+-ATPase activity and subunit content, additional experiments were performed where

active calpain and/or active MMP-2 were co-incubated with SL to study whether or not the effects seen in both ischemia and I/R were a direct result of protease activation or whether or not what is observed is part of a downstream mechanism where additional proteases are activated to impair Na^+-K^+-ATPase activity and degrade Na^+-K^+-ATPase subunits. These experiments also indicated whether or not calpain or MMP-2 activation are dependent or independent of each other and which protease is activated further downstream in the signal transduction mechanism leading to impaired Na^+-K^+-ATPase activity and subunit degradation as earlier studies have found that MMP-2 activation is dependent on calpain activation (Singh et al. 2012).

4. Hypotheses

1. Hearts subjected to ischemia exhibit impaired Na^+-K^+-ATPase activity, degradation of Na^+-K^+-ATPase subunits, and activation of proteolytic enzymes.

2. Hearts subjected to I/R exhibit impaired Na^+-K^+-ATPase activity, degradation of Na^+-K^+-ATPase subunits, and sustained activation of proteolytic enzymes.

3. Na^+-K^+-ATPase subunit degradation is more susceptible to calpain proteolytic activity than MMP-2 proteolytic activity.

III. MATERIALS AND METHODS

1. Perfusion and experimental protocol

Male Sprague-Dawley rats weighing 225-275 g were anaesthetized with a mixture of xylazine (9 mg/kg) and ketamine (90 mg/kg). Hearts were rapidly removed and cannulated to the Langendorff apparatus for retrograde perfusion with Krebs-Henseleit (K-H) buffer gassed with 95% O_2 mixed with 5% CO_2 at a rate of 10 mL/min. K-H buffer contained (in mmol/L): 120 NaCl, 25 $NaHCO_3$, 11 glucose, 4.8 KCl, 1.2 KH_2PO_4, 1.25 $MgSO_4$, 1.25 $CaCl_2$. The perfusion medium, pH 7.4, was maintained at a temperature of 37°C. The method for heart perfusion was the same as described elsewhere (Singh and Dhalla 2010; Singh et al. 2012) except that the heart was not stimulated electrically. A water-filled latex balloon was inserted in the left ventricle, after removal of both left and right atria, and connected to a pressure transducer (Model 1050BP; BioPac System Inc., Goleta, CA) to record left ventricular systolic and diastolic pressures. Left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP) were calculated using the Acknowledge 3.0.3 software for Windows (BioPac System Inc., Goleta, CA). The method for hemodynamic measurement was the same used by Singh and Dhalla (2010).

All hearts were stabilized for a period of 20 minutes before induction of ischemia and maintained at 37°C for all experimental groups. Global ischemia was induced by stopping the flow of K-H buffer for periods of 15, 30 and 60 minutes. In order to simulate I/R injury, global ischemia was induced for 45 minutes, followed by reperfusion for periods of 5, 10, 20, and 40 minutes. Control hearts were perfused with oxygenated medium for comparable periods. For studying the effects of calpain or MMP-2 inhibition

on I/R injury, the hearts were perfused for 10 minutes prior to inducing global ischemia and for 20 minutes upon initiating reperfusion with 10 μmol/L MDL28170 or 30 μmol/L doxycycline, respectively. The concentrations of these inhibitors were chosen as they have been shown to be effective in inhibiting their respective protease activities (Fert-Bober et al. 2008; Singh et al. 2012). At the end of the experiments, hearts were freeze-clamped in liquid N_2 and stored at -80°C for biochemical analysis.

2. Isolation of SL membrane

SL membranes were isolated from perfused hearts by previously described methods (Ostadal et al. 2004; Singh and Dhalla 2010). Hearts were homogenized in sucrose (600 mmol/L) and imidazole (10 mmol/L) solution and centrifuged at 12 000 g for 30 minutes after which the supernatant was collected, diluted witha KCl (160 mmol/L) and MOPS (20 mmol/L) buffer and centrifuged at 100 000 g for 60 minutes. The cytosolic fraction was collected as supernatant and used for the analysis of protease activity. The pellet thus obtained was suspended in KCl-MOPS buffer, which was then layered onto a solution made up of Tris-HCl (100 mmol/L), sodium pyrophosphate (50 mmol/L), KCl (300 mmol/L), and 30% sucrose. This was centrifuged for 90 minutes at 100 000 g which yielded a 3-layer sample where the white middle layer containing the SL membrane was carefully suctioned out and then centrifuged for 30 minutes at 100 000 g. The pellet of purified SL was suspended in a buffer containing 250 mmol/L sucrose and 10 mmol/L histidine and frozen at -80°C. All SL isolation procedures were performed at 4°C.

3. Incubation of SL with active proteases

In one set of experiments, SL preparation was isolated from unperfused hearts incubated for an hour at 37°C with or without active proteases. The following experimental groups incubated with active proteases using specific activity of 25 U (one U = 100 pmol/min at 37°C): control, where no active proteases were used; calpain (Biovision, Milpitas, CA); MMP-2 (Enzo Life Sciences, Farmingdale, NY); and both active proteases combined.

4. Measurement of SL Na^+-K^+-ATPase activity

Na^+-K^+-ATPase activity was measured by incubating SL at 37°C in assay tubes containing 10 mmol/L EGTA, 5 mmol/L NaN_3, and 6 mmol/L $MgCl_2$, with or without 100 mmol/L NaCl plus 10 mmol/L KCl in a total volume of 0.5 mL. The reaction was initiated by adding 80 mmol/L Tris-ATP, pH 7.4, for 10 minutes was terminated by adding 0.5 mL ice-cold 12% trichloroacetic acid; the reaction mixture was then centrifuged at 1000 g for 10 minutes. The supernatant was used for a phosphate assay using a spectrometer (Spectramax Plus, Molecular Devices, Sunnyvale, CA) where Na^+-K^+-ATPase activity was calculated as the difference between activities with and without Na^+ plus K^+. This method is the same as described earlier (Singh et al. 2012).

5. Western blot analysis

Protein content of Na^+-K^+-ATPase isoforms was determined by separating SL membranes (20 μg total protein/lane) on an 8% SDS-PAGE gel and electroblotting to polyvinylidene difluoride membranes (PVDF) according to the method used by Elmosehli et al. (2003).The Na^+-K^+-ATPase isoforms were detected using the following primary antibodies obtained from Upstate Biotechnologies (Lake Placid, CA): polyclonal

anti-α1 rabbit IgG, polyclonal anti-α2 rabbit IgG, polyclonal anti-β1 rabbit IgG, and polyclonal anti-β2 rabbit IgG. The secondary antibody used to detect all Na^+-K^+-ATPase subunits was a biotinylated goat anti-rabbit IgG (Bio-Rad, Hercules, CA). Membranes were incubated for 1 hour with streptavadin-conjugated horseradish peroxidase (1:5000) and then processed for chemiluminescence using a Pierce ECL kit (ThermoFisher Scientific, Waltham, MA). An imaging densitometer (GS800 Calibrated Densitometer, Bio-Rad, Hercules, USA) was used to scan the bands which were quantified using Quantity-One 4.6.9. To determine relative densities of proteins, blot radiograms were scanned and the scan values are expressed as a percentage of control taken as 100% in each group. For a loading control, PVDF membranes were incubated with ProSieve Blue Protein Staining Solution (Lonza, Rockland, ME) for one hour and then de-stained two times for 1 hour each using 30% ethanol. As there is currently no standard for loading control of sarcolemma due to it being a predominantly lipid structure, a reading of a non-descript band taken at 120 kDa was used as a comparative loading control.

6. Measurement of calpain activity

Calpain activity was measured in the isolated cytosolic fraction obtained from control and experimental hearts using a kit from Biovision (Milpitas, CA) according to the method employed by Singh et al. (2012). Samples were co-incubated for 1 hour at 37°C with reactive fluorogenic calpain substrate (Ac-LLY-AFC) and compared to a positive control of active calpain I. The negative control was sample and fluorogenic calpain coincubated with a calpain inhibitor (Z-LLY-FMK). The reaction was carried out in a 96-well plate where samples were read on a fluorescence microplate reader at 400 nm excitation and 505 nm emission (Molecular Devices, Sunnyvale, California). The

results are expressed as relative fluorescent units (RFU).

7. Measurement of MMP-2 activity

Gelatin zymography was used to determine MMP-2 activity present in the cytosolic fraction of isolated hearts as described elsewhere (Heussen and Dowdle 1980). Samples were prepared in non-reducing loading buffer and applied to an 8% polyacrylamide gel copolymerized with 1 mg/mL gelatin. Each lane was loaded with 24 μg of cytosolic protein. After electrophoresis, 2.5% Triton X-100 (3x20 min) was used to wash the gels to remove sodium dodecyl sulfate. The gels were rinsed 5x in incubation buffer made up of Tris-Hcl (50 mmol/L), $CaCl_2$ (5 mmol/L), NaCl (150 mmol/L) and 0.05% NaN_3. Gels were shaken slowly at 37°C for 48 hours and then stained in Coomassie blue for 1 hour. After staining, the gels were destained twice for 30 minutes each using a destaining solution made up of 10% isopropanol and 12% acetic acid. Zymograms were scanned using a GS800 Calibrated Densitometer (Bio-Rad, Hercules, USA) and the band intensities were measured using Quantity-One 4.6.9. MMP activities are expressed as a ratio to control.

8. Statistical Analysis

The values are expressed as mean ± SE and the differences between multiple groups were statistically evaluated by groups were evaluated by using analysis of variance (ANOVA) followed by a Student Newman-Keuls test using Prism 5 (Graphpad Software, Inc., La Jolla, California). A level of $p < 0.05$ was considered the threshold for statistical significance between the control and experimental groups as well as within the groups themselves.

IV. Results

1. Cardiac Dysfunction in Ischemic and I/R Hearts

In order to identify changes in cardiac function at different times of inducing global ischemia as well as at different times of reperfusion of the ischemic hearts, both LVDP and LVEDP were measured and the data are shown in Table 1. Upon the induction of global ischemia, LVDP was significantly decreased from control values (124.2 mmHg) to 10.3 mmHg, 37.0 mmHg, and 26.1 mmHg, at 15, 30, and 60 minutes, respectively. Corresponding LVEDP values were 8.7 mmHg, 35.5 mmHg, and 24.7 mmHg, indicating a marked decrease in cardiac function prominent in both diastolic relaxation and systolic contraction during global ischemia. Reperfusion of heart subjected to 45 min of global ischemia for 5, 10, 20, and 40 minutes showed that LVDP values were 91.9 mmHg, 94.6 mmHg, 94.7 mmHg, and 96.1 mmHg, whereas LVEDP values were 60.2 mmHg, 42.5 mmHg, 53.4 mmHg, and 60.1 mmHg, respectively (Table 1A). In view of the control values for LVDP (124 mmHg) and LVEDP (6 mmHg), ischemic hearts showed a marked recovery in LVDP but LVEDP remained increased upon reperfusion (Table 1B). Treatment of hearts with a calpain inhibitor (MDL28170) or a MMP-2 inhibitor (doxycycline) prevented the I/R-induced increase in LVEDP without affecting the recovery in LVDP (Table 1C).

Table 1. Alterations in cardiac function (LVDP and LVEDP) in hearts subjected to global ischemia for different times and reperfusion of the 45 min ischemic heart for different times, as well as ischemia reperfusion injury in hearts perfused in the absence or presence of protease inhibitors.

	Cardiac Function	
	LVDP (mm Hg)	**LVEDP (mm Hg)**
A. Time of global ischemia (min)		
Control	124.2 ± 3.9	4.8 ± 0.96
15 min	10.3 ± 5.0*	8.7 ± 4.6
30 min	37.0 ± 5.4*	35.5 ± 5.4*
60 min	26.1 ± 3.2*	24.7 ± 3.3*
B. Time of reperfusion (min) in 45 min ischemic hearts		
Control	122.9 ± 7.5	6.1 ± 1.0
0 min	35.2 ± 1.4*	34.4 ± 1.4*
5 min	91.9 ± 12.8*	60.2 ± 13.1*
10 min	94.6 ± 4.7*	42.5 ± 7.5*
20 min	94.7 ± 9.7*	53.4 ± 10.4*
40 min	96.1 ± 5.1*	60.1 ± 9.9*
C. Effect of protease inhibitors on I/R injury		
Control hearts	124.8 ± 5.6	7.2 ± 1.9
I/R hearts	95.4 ± 1.7*	77.7 ± 8.2*
MDL28170-treated I/R hearts	103.6 ± 6.1	38.8 ± 5.9#
Doxycycline treated I/R hearts	108.4 ± 4.6	55.7 ± 6.1#

Values are mean ± SE of 4-6 hearts in each group. (A) Hearts were subjected to global ischemia for 15, 30, or 60 min; (B) 45 min ischemic hearts were subjected to reperfusion for 5, 10, 20, and 40 min; (C) I/R injury was induced by subjecting hearts to 30 min global ischemia followed by 45 min reperfusion in the absence (I/R) and presence of MDL28170 (10 μmol/L) or doxycycline (30 μmol/L). LVDP – left ventricular developed pressure; LVEDP – left ventricular end diastolic pressure. * - Significantly different ($P<0.05$) from Control in A, B and C; # - significantly different ($P<0.05$) from I/R in C.

2. Time-course alterations of protease and Na^+-K^+-ATPase activities and subunit degradation

Protease and Na^+-K^+-ATPase activities were measured at different times of ischemia as well as upon reperfusion after 45 min of ischemia and the results are shown in Figure 1. During ischemia, Na^+-K^+-ATPase activity gradually decreased over time which becomes statistically significant at 60 min of ischemia. The activity of Na^+-K^+-ATPase in ischemic hearts was markedly depressed upon reperfusion for 5 to 40 min. Calpain activity measured in the cytosol was markedly increased as early as 15 min into ischemia and remained significantly elevated throughout the ischemic period. Calpain activity was elevated upon reperfusing the ischemic hearts in comparison to the control values at all times tested. MMP-2 activity gradually increased during ischemia over time and becomes significantly elevated after 30 min and 60 min. MMP-2 activity was increased in ischemic hearts in a biphasic manner during 5 to 40 min of reperfusion compared to control values (Figure 1). The data in Figures 2 and 3 show the degradation of Na^+-K^+-ATPase subunits during ischemia and reperfusion. No change was found in the α1- and α2-subunits during ischemia; however, upon reperfusion, a significant decrease in the protein content for both α-1 and α-2 subunits started as early as 10 min into reperfusion. The level of degradation of Na^+-K^+-ATPase α1- and α2-subunits remained relatively stable during 20 and 40 min of reperfusion. Na^+-K^+-ATPase β1- and β2-subunits degradation occurred during 5 to 40 min into ischemia. The most sensitive of the Na^+-K^+-ATPase subunits was β2, which showed a marked decrease in its levels as early as 5 min post-reperfusion when compared to the depression in β1-subunit degradation.

Figure 1: SL Na^+-K^+-ATPase activity (a,d), as well as cytosolic calpain (b,e), and MMP-2 activities (c,f) from hearts collected at 15, 30, and 60 min of ischemia and hearts reperfused at 5, 10, 20 and 40 min after 45 min of ischemia. Each value is a mean ± SE of 6 experiments in each group; * - Significantly different ($P < 0.05$) in comparison to control; # - significantly different ($P < 0.05$) compared to 40 min reperfusion.

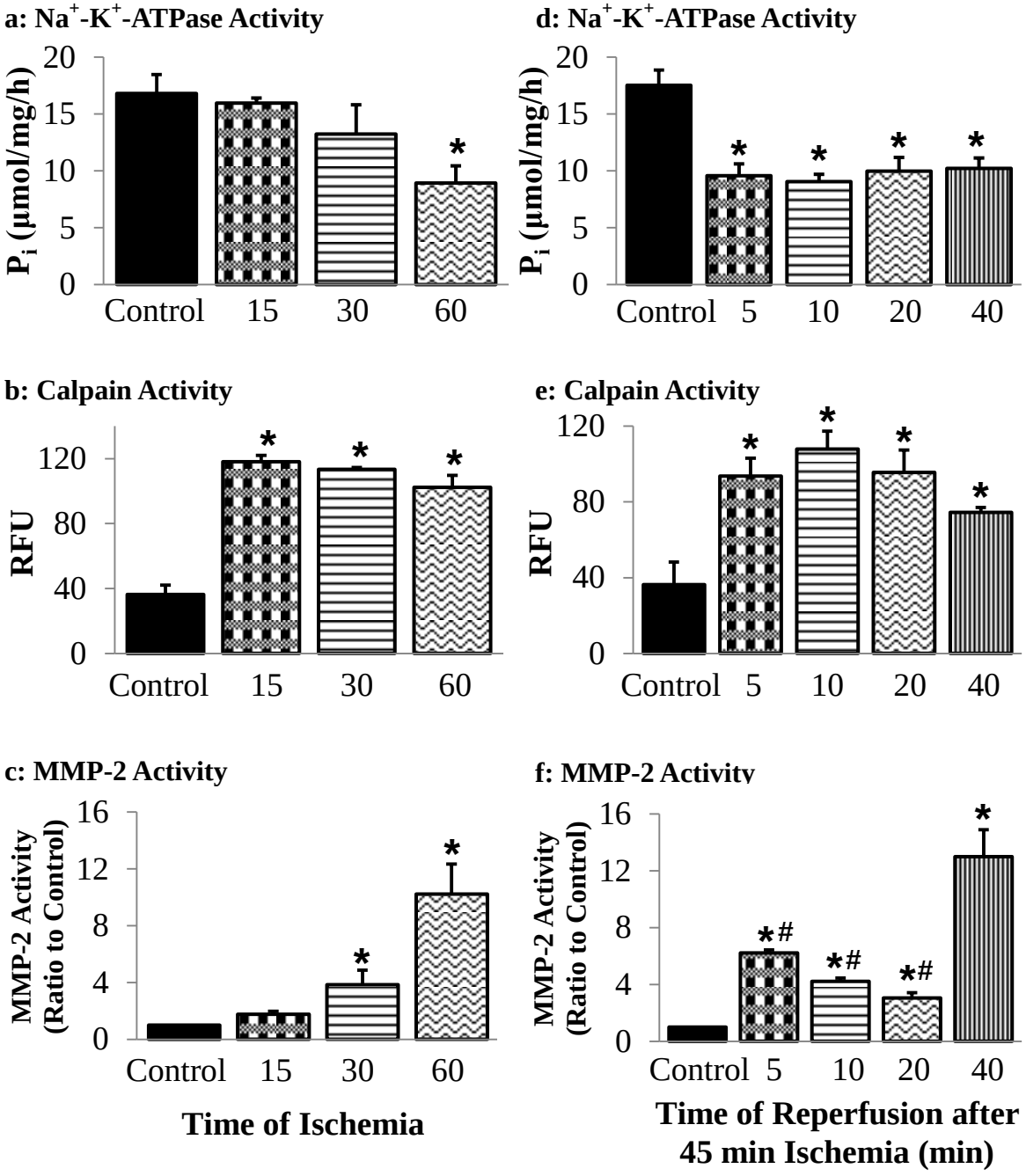


Figure 2: Protein content of Na^+-K^+-ATPase α1-(a,c) and α2-subunits (b,d) in SL preparations from hearts collected at 15, 30, and 60 min of ischemia and hearts reperfused at 5, 10, 20 and 40 min after 45 min of ischemia. Each value is a mean ± SE of 6 experiments in each group; * - Significantly different ($P < 0.05$) in comparison to control.

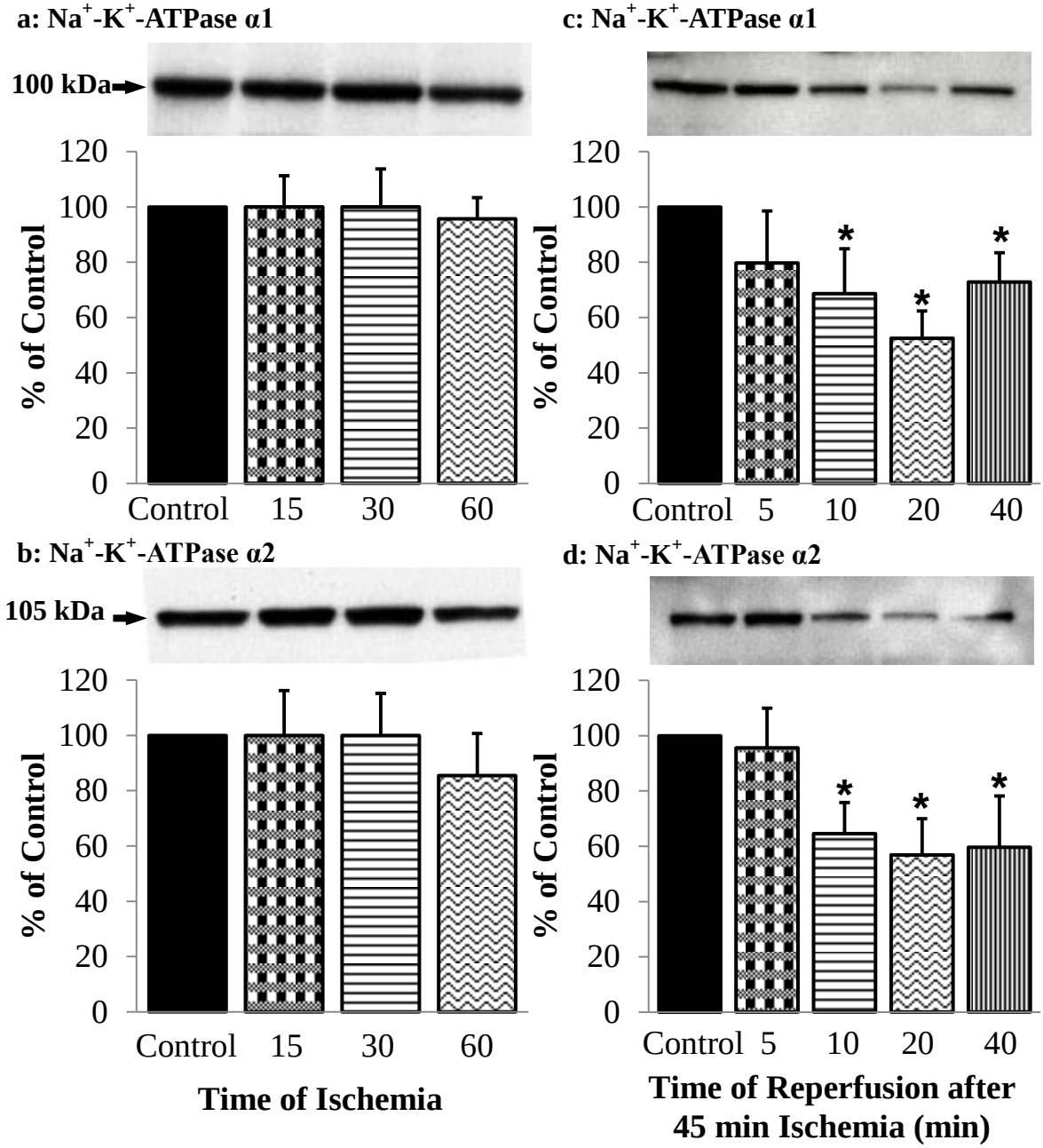
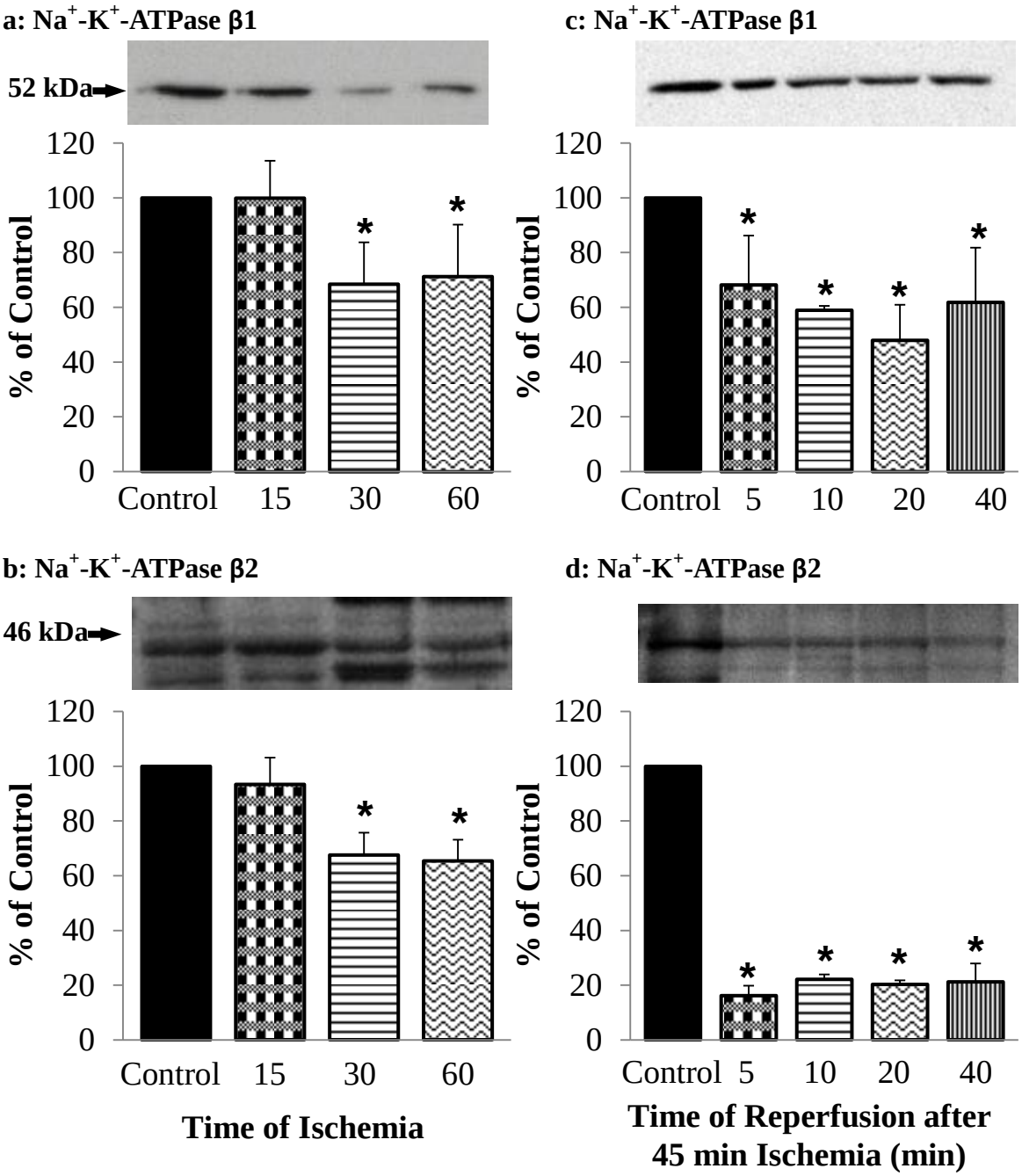


Figure 3: Protein content of Na^+-K^+-ATPase β1- (a,c) and β2-subunits (b,d) in SL preparations from hearts collected at 15, 30, and 60 min of ischemia and hearts reperfused for 5, 10, 20 and 40 min after 45 min of ischemia. Each value is a mean ± SE of 6 experiments in each group; * - Significantly different ($P < 0.05$) in comparison to control.



3. Effect of perfusion with calpain and MMP-2 inhibitor treatment on I/R-induced changes in protease activities as well as Na^+-K^+-ATPase activities and subunit concentration

Both MDL28170, a calpain-specific inhibitor, and doxycycline, and MMP-2 inhibitor, were found to affect I/R-induced changes in Na^+-K^+-ATPase and the results are shown in Figures 4 and 5. I/R injury caused a decrease in Na^+-K^+-ATPase activity which was significantly attenuated by calpain inhibition with MDL28170 but not by MMP inhibition with doxycycline. Mg^{2+}-ATPase measurements were done to ensure that the ATPase activity that was measured was specific for Na^+-K^+-ATPase. There were no significant differences between any groups in Mg^{2+}-ATPase when compared to control (Figure 4). Cytosolic calpain activity was significantly increased due to I/R injury; this effect was significantly decreased by MDL28170, unlike doxycycline. MMP-2 activity was also found to be significantly increased in I/R injury; perfusion of hearts with both calpain inhibitor, MDL28170 and MMP inhibitor, doxycycline, significantly attenuated the I/R-induced changes in MMP-2 activity (Figure 4). Na^+-K^+-ATPase subunit concentrations were also measured using western blotting to evaluate the effect of protease inhibition on Na^+-K^+-ATPase subunits. MDL28170 and doxycycline were both found to be effective in attenuating Na^+-K^+-ATPase subunit degradation for α-1, β-1, and β-2 due to I/R injury; however only MDL28170 was able to significantly attenuate the degradation of Na^+-K^+-ATPase α-2 (Figure 5).

Figure 4: SL Na^+-K^+-ATPase (a) and Mg^{2+}-ATPase (b) activities as well as cytosolic calpain (c) and MMP-2 (d) activities of hearts subjected to 30 min ischemia and 30 min reperfusion in the absence or presence of calpain inhibitor, MDL28170 (MDL), or MMP-2 inhibitor, doxycycline (Dox). Each value is a mean ± SE of 6 experiments in each group; * - Significantly different ($P < 0.05$) from control; # - Significantly different ($P < 0.05$) from I/R without any treatment.

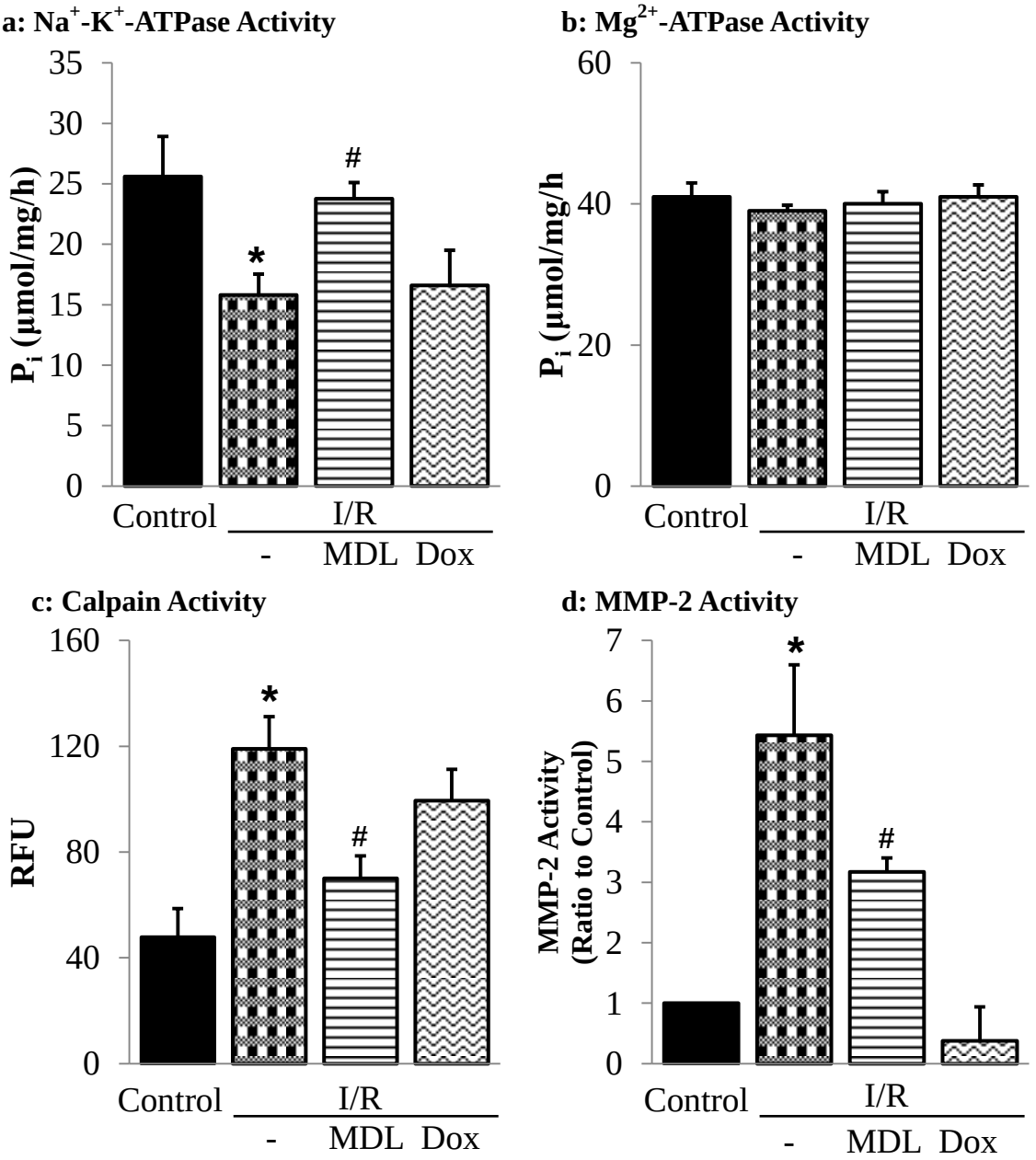
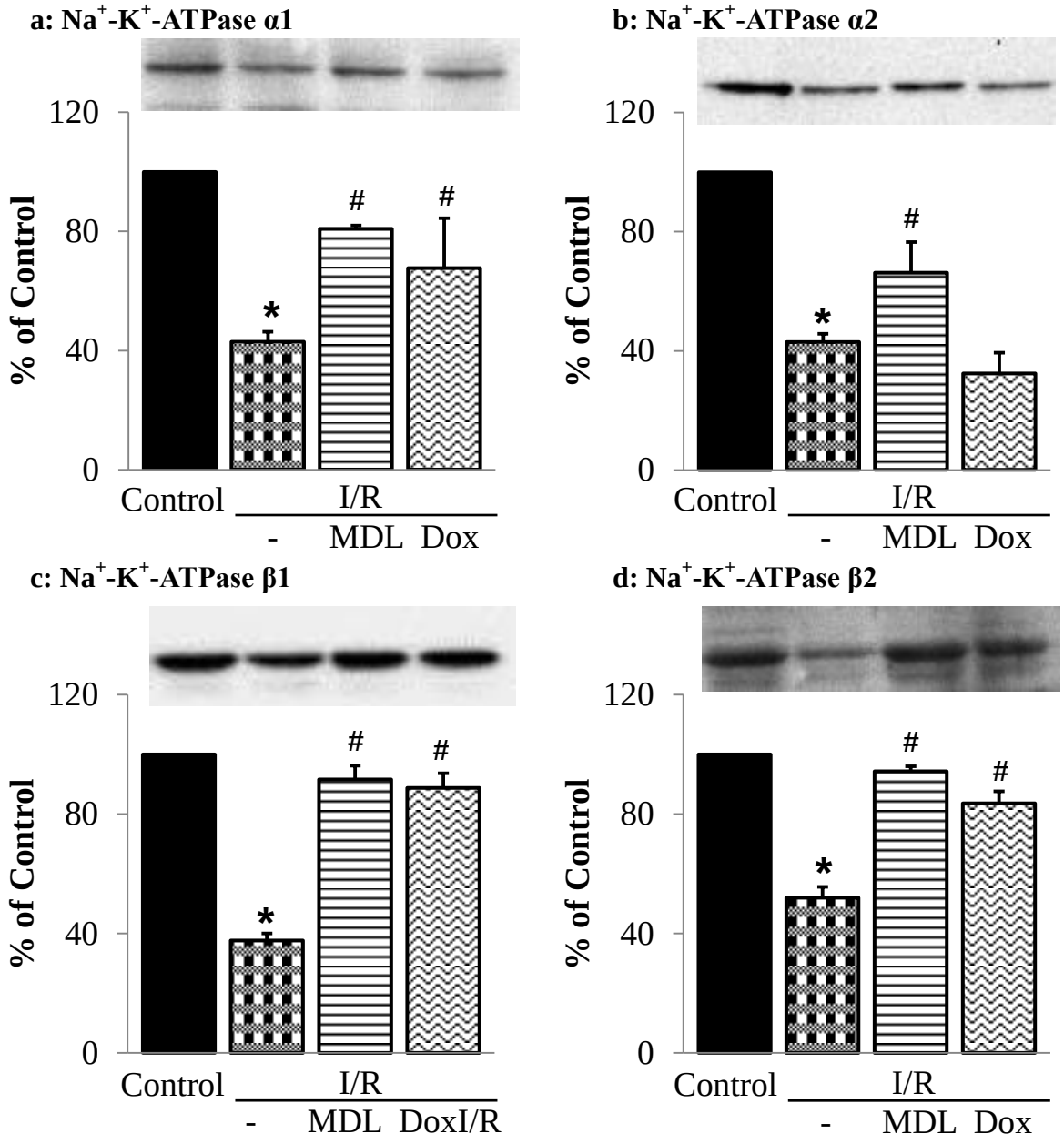


Figure 5: Protein content of Na^+-K^+-ATPase subunits in SL preparations from hearts subjected to ischemic reperfusion (I/R) injury in the absence or presence of calpain inhibitor, MDL28170 (MDL), or MMP-2 inhibitor, doxycycline (Dox). Representative blots for each Na^+-K^+-ATPase subunits are shown in different panels. Each value is a mean ± SE of 6 experiments in each group; * - Significantly different ($P < 0.05$) from control; # - Significantly different ($P < 0.05$) from I/R without any treatment.

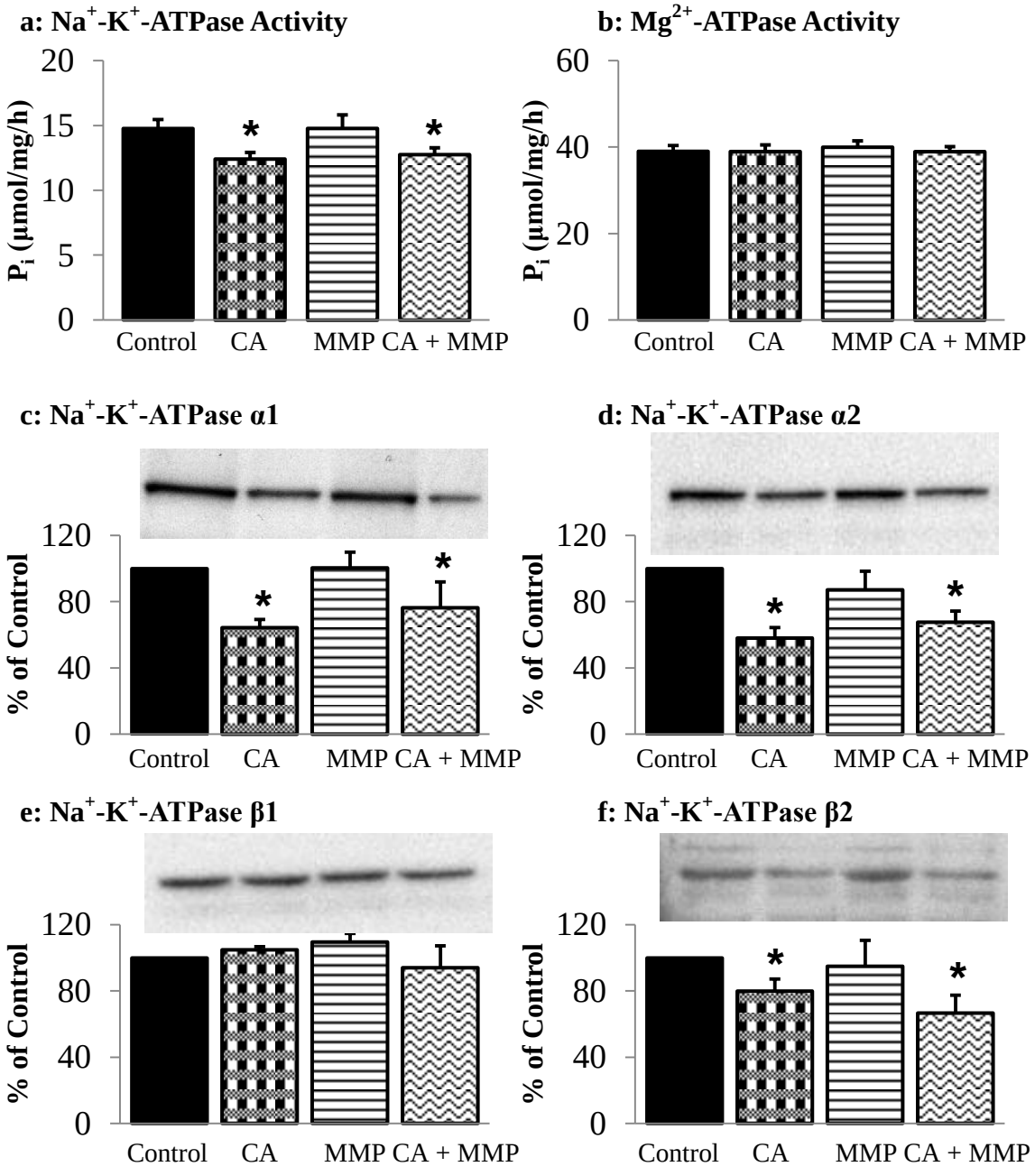


4. Effects of active calpain and MMP-2 on Na^+-K^+-ATPase activity and subunits in isolated SL membrane

In order to test whether the depression in Na^+-K^+-ATPase is caused by calpain and/or MMP-2 directly, isolated SL membrane was incubated with these proteases and the results are shown in Figure 6. Active calpain was found to significantly depress Na^+-K^+-ATPase activity and decrease concentrations of Na^+-K^+-ATPase α-1, α-2, and β-2 when compared to untreated isolated hearts. Mg^{2+}-ATPase activity remained unchanged amongst the experimental groups. Active MMP-2 was found to have no direct effect on Na^+-K^+-ATPase or any of its subunits. Although incubation of SL membrane with both active calpain and MMP-2 decreased Na^+-K^+-ATPase activity, and degraded Na^+-K^+-ATPase α-1, α-2, and β-2, the effects of calpain were not amplified by MMP-2. Furthermore, protein content for the Na^+-K^+-ATPase β1-subunit was not decreased by incubation of SL membrane with either protease (Figure 6).

Figure 6: Na^+-K^+-ATPase activity, Mg^{2+}-ATPase activity, and Na^+-K^+-ATPase subunit protein content in isolated SL treated with or without active calpain (CA) and/or active MMP-2 (MMP). Representative blots for each Na^+-K^+-ATPase subunit are shown in different panels. Each value is a mean ± SE of 4 experiments in each group;

* - Significantly different ($P < 0.05$) from control.



V. Discussion

1. Cardiac dysfunction due to global ischemia and reperfusion

By employing isolated rat hearts perfused at a constant flow without any electrical stimulation to maintain heart rate, we have observed that LVDP was depressed by 70 to 90% and LVEDP was increased by 2 to 7 fold when inducing global ischemia for 10 to 60 minutes (Table 1). Furthermore, reperfusion of 45 min ischemic hearts for 5 to 40 min decreased the recovery of LVDP by 23 to 27% and increased the LVEDP by 8 to 12 fold. This was done to better understand the endogenous pace-maker mechanism of the heart which relies heavily on ion transport across the membrane without any potential artifacts that may occur with artificial stimulation, especially since stimulation is sustained during ischemia in many I/R models which does not occur clinically during ischemia. These observations indicating cardiac dysfunction due to global ischemia and I/R injury are in agreement with different reports from other laboratories (Elmoselhi et al. 2003; Ostadal et al. 2004; Saini and Dhalla 2005; Saini et al. 2005; Singh and Dhalla 2010; Singh et al. 2012; Temsah et al. 1999). However, it is pointed out that the recovery of LVDP in ischemic hearts was much higher and the increase in LVEDP was much greater upon reperfusion in comparison to these previous reports which employed electrically stimulated heart preparations. Differences in the time of development of cardiac dysfunction due to myocardial ischemia and the start of I/R injury in hearts perfused at constant pressure have also been observed (Singh et al. 2008). Thus it appears that alterations in both LVDP and LVEDP during the development of cardiac dysfunction in ischemic hearts as well as during the process of recovery upon reperfusion may depend upon the experimental conditions employed for perfusing the isolated heart preparations.

2. Depression in Na^+-K^+-ATPase activity due to global ischemia and reperfusion

We have observed that SL Na^+-K^+-ATPase activity was significantly depressed in hearts subjected to prolonged (60 min) global ischemia as well as in 45 min ischemic hearts upon reperfusion for 5 to 40 min (Fig 1). These results are consistent with cardiac reports showing depression in Na^+-K^+-ATPase activity due to I/R injury (Elmoselhi et al. 2003; Ostadal et al. 2004; Singh and Dhalla 2010; Singh et al. 2012) and are not due to any non-specific changes in the SL membrane because Mg^{2+}-ATPase activity was not altered upon reperfusion of the ischemic hearts. In view of the well-known function of Na^+-K^+-ATPase to serve as a Na^+-pump, a depression in the SL Na^+-K^+-ATPase activity due to I/R injury can be seen to increase the intracellular concentration of Na^+, which, in turn, would result in the development of intracellular Ca^{2+}-overload due to stimulation of the Na^+-Ca^{2+}-exchanger operating on reverse mode (Dhalla et al. 2007; Saini and Dhalla 2005; Saini et al. 2005). The occurrence of intracellular Ca^{2+}-overload has been demonstrated to produce cardiac dysfunction in ischemic heart disease (Dhalla et al. 1998; Dhalla et al. 2000; Dhalla et al. 2007). The observed depression in Na^+-K^+-ATPase activity due to I/R injury can be seen to produce cardiac dysfunction indirectly through the induction of intracellular Ca^{2+}-overload. However, it should be noted that cardiac dysfunction in hearts subjected to 15 to 30 min of ischemia was not associated with any significant depression in Na^+-K^+-ATPase activity. Thus, it is likely that mechanisms other than the inhibition of Na^+-K^+-ATPase may also be participating in inducing cardiac dysfunction due to ischemic injury.

3. Alterations in Na^+-K^+-ATPase subunits due to global ischemia and reperfusion

The results in this study have revealed marked changes in protein content for different subunits of Na^+-K^+-ATPase in hearts subjected to global ischemia and reperfusion. Alterations in the subunit composition of Na^+-K^+-ATPase due to I/R injury in the heart have also been reported earlier (Ostadal et al. 2004). It is noteworthy to point out that degradation of α1-, α2-, β1- and β2-subunits was associated with a depression in Na^+-K^+-ATPase activity upon reperfusion of the ischemic hearts for 5 to 40 min (Fig 2 and 3). However, while both β1- and β2-subunits and Na^+-K^+-ATPase activity was decreased in ischemic hearts reperfused for 5 min, no changes in α1- and α2-subunits of Na^+-K^+-ATPase was evident. Furthermore, global ischemia for 30 min decreased β1- and β2- subunit content without any changes in Na^+-K^+-ATPase activity as well as α1- and α2-subunits. On the other hand, global ischemia for 60 min showed depression in Na^+-K^+-ATPase activity and β1- and β2-subunits without affecting α1- and α2-subunits of Na^+-K^+-ATPase. These observations support the view that β1- and β2-subunits of Na^+-K^+-ATPase are more sensitive to global ischemia and I/R injury compared to α1- and α2-subunits. In addition, while alterations in the composition of Na^+-K^+-ATPase subunits can be seen to explain the observed depression in Na^+-K^+-ATPase activity in I/R hearts, other mechanisms such as oxidative stress have been shown to decrease Na^+-K^+-ATPase activity due to oxidation of its functional groups (Dhalla et al. 2000).

4. Activation of calpain and MMP-2 due to global ischemia and reperfusion

The observed activation of both calpain and MMP-2 in hearts subjected to global ischemia and reperfusion is in agreement with previous reports showing the effect of I/R injury (Chohan et al. 2006; Schulze et al. 2003; Schulz 2007; Singh and Dhalla 2010; Singh et al. 2012). It may be noted that the activation of calpain was seen during 15 to 60 min of inducing global ischemia whereas the activation of MMP-2 became apparent at 30 to 60 min of ischemia (Fig 5). The sustained activation of calpain in ischemia indicates that calpain does not necessarily require Ca^{2+}-overload caused by reperfusion in order to become active. It has been found, that although a significant increase in Ca^{2+} does occur during the reperfusion period of I/R injury, there are increased levels of both cytoplasmic and mitochondrial Ca^{2+} within the myocardium, particularly in the early phase of ischemia (Henry PD et al. 1977; Sybers et al. 1983). It has also been shown that the level of intracellular Ca^{2+} corresponds to mechanical changes induced by ischemia (Henry PD et al. 1977). In addition, and in agreement with a study done by Cheung et al. (2000), as the duration of ischemia increases, so does the activity of MMP-2, although our data contributes the novel finding that this increase in MMP-2 proteolytic activity is occurring in the cytosol in addition to the extracellular matrix. On the other hand, the activation of MMP-2 due to I/R injury was biphasic, an increase at 5 min followed by a significant decrease at 10 and 20 min, and then an increase at 40 min. Such a differentiated pattern of changes in the activation of calpain and MMP-2 in ischemic as well as reperfused hearts may be due to differences in the mechanisms of activation of these proteases. While activation of calpain in the I/R heart has been suggested to be due to the occurrence of Ca^{2+}-overload (Singh and Dhalla 2010), the activation of MMP-2 has been

indicated to be due to the development of oxidative stress (Schulz 2007). Activation of MMP-2 in I/R hearts has also been suggested to be a result of the activation of calpain (Singh et al. 2012). Nonetheless, activation of the both calpain and MMP-2 can be seen to degrade different subunits of Na^+-K^+-ATPase in a time-dependent manner. Since the incubation of SL membrane with calpain, unlike MMP-2, was found to alter Na^+-K^+-ATPase activity and its subunit composition, it is likely that the effects of calpain activation are of a direct nature whereas that of MMP-2 activation may be of an indirect nature. Furthermore, changes in Na^+-K^+-ATPase subunit composition in I/R hearts may not only be due to the activation of protease because I/R has been shown to produce dramatic changes in gene expression for these subunits (Ostadal et al. 2003).

5. Effects of protease inhibition in ischemic and reperfused hearts

It is important to note that the inhibitors in this study used to evaluate the effect of calpain and MMP-2 in ischemia-reperfusion injury are non-specific. MDL28170 has been reported to inhibit cathepsin L in monkey kidney epithelial cells when infected with the severe acute respiratory syndrome (SARS) virus (Simmons et al. 2005); however this pharmacological inhibitor has not yet been found to inhibit cathepsin L in the heart of humans. Although doxycycline is a non-specific inhibitor of MMPs, it was chosen as it is currently the only FDA approved MMP inhibitor availably clinically due to its lack of severe side effects (Dorman et al. 2007). Its primary use is a tetracycline that acts as an antibacterial agent by inhibiting protein synthesis (Clark and Chang 1965). Although doxycycline has been shown to be a broad-spectrum MMP inhibitor, only MMP-2 has been shown to be present inside the cell whereas the other MMPs are extracellular proteases (Wang et al. 2002). Since the experiments evaluating MMP activity were

performed using isolated cytosol, the influence of extracellular MMP inhibition is highly improbable. In addition, doxycycline has not yet been shown to inhibit other intracellular proteases.

Treatment of hearts with MDL28170 and doxycycline were found to attenuate I/R-induced increases in LVEDP without further improving the recovery of LVDP in I/R hearts (Table 1). The ineffectiveness of these protease inhibitors to further improve the recovery of LVDP is in contrast to the earlier observation in which electrically stimulated heart preparations were employed and I/R produced a marked depression in LVDP and both these agents enhanced the recovery of the heart (Singh et al. 2012). It is also noteworthy that calpain inhibition attenuated the I/R-induced depressions in Na^+-K^+-ATPase activity and all its subunits (Figure 6). On the other hand, MDL28170 reduced I/R induced alterations in α1-, β1-, and β2-subunits without affecting the I/R induced changes in α2-subunit of Na^+-K^+-ATPase as well as its activity. These observations suggest that the inhibition of Na^+-K^+-ATPase activity in I/R hearts may not be entirely due to the activation of calpain and MMP-2, although it has been shown that inhibition of calpain indirectly impairs MMP activity (Ali et al. 2012; Singh et al. 2012). It is likely that activation of other proteases (Müller et al. 2012) may also be involved in changing the composition of Na^+-K^+-ATPase and its activity in the I/R heart. Varying degrees of inhibitory effects of MDL28170 and doxycycline on I/R-induced activations of calpain and MMP-2 indicate the non-specific nature of these agents. Nonetheless, the overall preventative effects of both MDL28170 and doxycycline support the role of calpain and MMP activation in the pathogenesis of cardiac dysfunction due to I/R injury. Such beneficial effects of these agents on the I/R-induced injury to the heart may not be limited

to the attenuation of SL changes because alterations in other subcellular organelles such as the sarcoplasmic reticulum have been shown to occur as a consequence of protease activation due to I/R injury (Chohan et al. 2006; Singh and Dhalla 2010). When comparing the gradual activation of MMP-2 with the relatively immediate activation of calpain, it is possible to consider that calpain activation may be mediating MMP-2 activation further downstream. Thus, inhibiting calpain could be more effective in preventing intracellular damage cause by both calpain and MMP-2.

VI. Future Directions

There is currently no standard reperfusion therapy treatment that is used in a clinical setting to reduce I/R injury. Currently, the only FDA-approved pharmaceutical shown to effectively specifically target calpain-1 in humans is called rosiglitazone (Randriamboavonjy et al. 2008); however its efficacy in preventing subcellular remodelling in cardiovascular disease is currently unknown. Its ability in inhibiting calpain-induced subcellular remodelling needs to be evaluated by performing studies in animals to evaluate its ability to prevent subcellular remodelling damage. In addition, further studies need to be done in order to ascertain its ability to either directly or indirecty inhibit MMP-2 activation. If the initial animal studies prove successful, the final step would involve multi-center clinical trials using this inhibitor where it is introduced slighty prior to reperfusion and administered directly to the infarct site in order to minimize reperfusion damage.

VII. Conclusions

From the results described in this study, the following conclusions can be drawn:

1. Subjecting the heart to a prolonged period of ischemia (60 min) induced activation of both calpain and MMP-2, depression in Na^+-K^+-ATPase activity and protein content of β1- and β2-subunits of Na^+-K^+-ATPase, as well as cardiac dysfunction. However, increases in calpain activity and decreases in LVDP at early periods of ischemia (15 min) were not associated with any changes in Na^+-K^+-ATPase activity or subunit composition.

2. Reperfusion of the 45 min ischemic heart for 5 to 40 min produced a marked recovery of LVDP, increases in LVEDP, calpain and MMP-2 activities, as well as depression in the Na^+-K^+-ATPase activity and protein content of α1-, α2-, β1- and β2 subunits of Na^+-K^+-ATPase except that protein content of α1- and α2- subunits were not affected.

3. Treatment of I/R hearts with a calpain inhibitor (MDL28170) or MMP-2 inhibitor (doxycycline) prevented the I/R induced changes in LVEDP, Na^+-K^+-ATPase activity and subunit composition except that I/R induced depressions in Na^+-K^+-ATPase activity as α2-subunit content was not altered by doxycycline.

4. Incubation of SL membranes with calpain, unlike MMP-2, under *in vitro* conditions decreased Na^+-K^+-ATPase activity and protein content for α1-, α2- and β2-subunits of Na^+-K^+-ATPase.

5. Although activation of both calpain and MMP-2 may alter the subunit composition of Na^+-K^+-ATPase due to I/R injury, α1- and α2-subunit are more resistant compared to β1- and β2-subunits.

6. Alterations in Na^+-K^+-ATPase activity and subunit composition may be due to a direct action of calpain on the enzyme whereas these changes due to the activation of MMP-2 appear to be of an indirect nature.

VIII. References

Akula A, Veeravalli KK, Raju SP, Kota MK. Studies on the signal cascade mechanism mediating the cardioprotective actions of bradykinin. Pharmazie 2002; 57:332–336.

Ali MA, Cho WJ, Hudson B, Kassiri Z, Granzier H, Schulz R. Titin is a target of matrix metalloproteinase-2: Implications in myocardial ischemia/reperfusion injury. Circulation 2010; 122:2039-2047.

Ali MA, Schulz R. Activation of MMP-2 as a key event in oxidative stress injury to the heart. Front in Biosc 2009; 14:699-716.

Ali MA, Stepanko A, Fan X, Holt A, Schulz R. Calpain inhibitors exhibit matrix metalloproteinase-2 inhibitory activity. Biochem Biophys Res Commun 2012 [Epub ahead of print].

Annapurna A, Kumar VK. Effect of inhibition of angiotensin converting enzyme by ramipril and aminopeptidase P by 2-mercaptoethanol in rats with experimental myocardial infarction. Indian J Pharmacol 2001; 33:72–76.

Arora RC, Hess ML. Effect of reduced oxygen intermediates on sarcolemmal muscarinic receptors from canine heart. Biochem Biophys Res Commun 1985; 130:133-140.

Asemu G, Dent M, Singal T, Dhalla NS, Tappia PS. Differential changes in phospholipase D and phosphatidate phosphohydralase activities in ischemia-reperfusion of rat heart. Arch Biochem Biophys 2005; 436:136–144.

Asemu G, Tappia PS, Dhalla NS. Identification of the changes in phospholipase C isozymes in ischemic-reperfused rat heart. Arch Biochem Biophys 2003; 411:174–182.

Askenasy N, Vivi A, Tassini M, Navon G, Farkas DL. NMR spectroscopic characterization of sarcolemmal permeability during myocardial ischemia and reperfusion. J Mol Cell Cardiol 2001; 33:1421–1423.

Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 2002; 115:3719-3727.

Baumgarten G, Knuefermann P, Kalra D, et al. Load-dependent and -independent regulation of proinflammatory cytokine and cytokine receptor gene expression in the adult mammalian heart. Circulation 2002; 105:2192–2197.

Berry RG, Despa S, Fuller W, Bers DM, Shattock MJ. Differential distribution and regulation of mouse Na^+/K^+-ATPase α1 and α2-subunits in T-tubule and surface sarcolemmal membranes. Cardiovasc Res 2007; 73:92-100.

Bers DM. Cardiac excitation-contraction coupling. Nature 2002; 415: 198-205.

Bers DM. Calcium cycling and signaling in cardiac myocytes. Annu Rev Physiol 2008; 70: 23-49.

Bhuiyan MS, Fukunaga K. Activation of HtrA2, a mitochondrial serine protease mediates apoptosis: current knowledge on HtrA2 mediated myocardial ischemia/reperfusion injury. Cardiovasc Ther 2008; 26:224-232.

Brilla CG, Maisch B. Regulation of the structural remodeling of the myocardium from hypertrophy to heart failure. Eur J Biochem 1994; 15:45–52

Cardone RA, Casavola V, Reshkin SJ. The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis. Nat Rev Canc 2005; 5:786-795.

Chae SU, Ha KC, Piao CS, Chae SW, Chae HJ. Estrogen attenuates cardiac ischemia-reperfusion injury via inhibition of calpain-mediated bid cleavage. Arch Pharm Res 2007; 30:1225-1235.

Chavakis E, Urbich C, Dimmeler S. Homing and engraftment of progenitor cells: a prerequisite for cell therapy. J Mol Cell Cardiol 2008; 45:514–522.

Cheng XW, Obata K, Kuzuya M, et al. Elastolytic cathepsin induction/activation system exists in myocardium and is upregulated in hypertensive heart failure. Hypertension. 2006; 48:979–987.

Cheung P, Sawicki G, Wozniak M, et al. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. Circulation 2000; 101:1833-1839.

Chohan PK, Singh RB, Dhalla NS, Netticadan T. L-arginine administration recovers sarcoplasmic reticulum function in ischemic reperfused hearts by preventing calpain activation. Cardiovasc Res 2006; 69:152–163.

Clark JMJ, Chang AY. Inhibitors of transfer of amino acids from aminoacyl soluble ribonucleic acid to proteins. J Biol Chem 1965; 240:4734-4739.

Communal C, Sumandea M, de Tombe P, Narula J, Solaro RJ and Hajjar RJ. Functional consequences of caspase activation in cardiac myocytes. Proc Natl Acad Sci USA 2002; 99:6252–6256.

Cuervo AM, Wong ES, Martinez-Vicente M. Protein degradation, aggregation, and misfolding. Mov Disord 2010; 25 Suppl 1:S49-S54.

Davis GE, Martin BM. A latent MR 94,000 gelatin-degrading metalloproteinase induced during differentiation of HL-60 promyelocytic leukemia cells: a member of the collagenase family of enzymes. Cancer Res 1990; 50:1113-1120.

Decker RS, Poole AR, Griffin EE, Dingle JJ. Altered distribution of lysosomal cathepsin D in ischemic myocardium. J Clin Invest 1997; 59:911–921.

Decker RS, Wildenthal K. Lysosomal alterations in hypoxic and reoxygenated hearts. I. Ultrastructural and cytochemical changes. Am J Pathol 1980; 98:425-444.

Dell'Italia LJ, Meng QC, Balcells E, et al. Compartmentalization of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. J Clin Invest 1997; 100:253–258.

Dendorfer A, Wolfrum S, Wellhoner P, Korsman K, Dominiak P. Intravascular and interstitial degradation of bradykinin in isolated perfused rat heart. Br J Pharmacol 1997; 122:1179–1187.

Deschamps AM, Yarbrough WM, Squires CE, et al. Trafficking of the membrane type-1 matrix metalloproteinase in ischemia and reperfusion: relation to interstitial membrane type-1 matrix metalloproteinase activity. Circulation 2005; 111:1166–1174.

Deschamps AM, Zavadzkas J, Murphy RL, et al. Interruption of endothelin signaling modifies membrane type 1 matrix metalloproteinase activity during ischemia and reperfusion. Am J Physiol Heart Circ Physiol 2008; 294:H875-H883.

Despa S, Lingrel JB, Bers DM. Na/K-ATPase α2-isoform preferentially modulates Ca transients and sarcoplasmic reticulum Ca release in cardiac myocytes. Cardiovasc Res 2012 [Epub ahead of print].

Dhalla NS, Panagia V, Singal PK, Makino N, Dixon IM, Eyolfson DA. Alterations in heart membrane calcium transport during the development of ischemia-reperfusion injury. J Mol Cell Cardiol 1988; 20:S3–S13.

Dhalla NS, Golfman L, Takeda S, et al. Evidence for the role of oxidative stress in ischemia heart disease : a brief reiew. Can J Cardiol 1999 ; 15 :587-593.

Dhalla NS, Saini HK, Tappia PS, Sethi R, Mengi SA, Gupta SK. Potential role and mechanisms of subcellular remodeling in cardiac dysfunction due to ischemic heart disease. J Cardiovasc Med (Hagerstown) 2007; 8:238-250.

Dhalla NS, Temsah RM, Netticadan T. Role of oxidative stress in cardiovascular diseases. J Hypertens 2000; 18:655-673.

Diwan A, Dibbs Z, Nemoto S, et al. Targeted overexpression of noncleavable and secreted forms of tumor necrosis factor provokes disparate cardiac phenotypes. Circulation 2004; 109:262–268.

Dixon IM, Kaneko M, Hata T, Panagia V, Dhalla NS. Alterations in cardiac membrane Ca^{2+} transport during oxidative stress. Mol Cell Biochem 1990; 99:125-133.

Domenech RJ, Sanchez G, Donoso P, Parra V, Macho P. Effect of tachycardia on myocardial sarcoplasmic reticulum and Ca^{2+} dynamics: a mechanism for preconditioning? J Mol Cell Cardiol 2003; 35:1429–1437.

Donato M, D'Annunzio V, Buchholz B, et al. Role of matrix metalloproteinase-2 in the cardioprotective effect of ischaemic postconditioning. Exp Physiol 2010; 95:274-281

Donnini S, Monti M, Roncone R, et al. Peroxynitrite inactivates human-tissue inhibitor of metalloproteinase-4. FEBS Lett 2008; 582:1135-1140.

Dorman G, Kocsis-Szommer K, Spadoni C, Ferdinandy P. MMP inhibitors in cardiac disease: an update. Recent Pat Cardiovasc Drug Discov 2007; 2:186-194.

Elmoselhi AB, Lukas A, Ostadal P, Dhalla NS. Preconditioning attenuates ischemia-reperfusion-induced remodeling of Na+-K+-ATPase in hearts. Am J Physiol Heart Circ Physiol 2003; 285:H1055-H1063.

Ergul A, Walker CA, Goldberg A, et al. ET-1 in the myocardial interstitium: relation to myocyte ECE activity and expression. Am J Physiol Heart Circ Physiol 2000; 278:H2050–H2060.

Ersahin C, Euler DE, Simmons WH. Cardioprotective effects of aminopeptidase P inhibitor apstatin: studies on ischemia/reperfusion injury in the isolated rat heart. J Cardiovasc Pharmacol 1999; 34:604–611.

Everts V, Korper W, Hoeben KA, et al. Osteoclastic bone degradation and the role of different cysteine proteinases and matrix metalloproteinases: differences between calvaria and long bone. J Bone Miner Res 2006; 2:1399–1408.

Felbor U, Dreier L, Bryant RA, Ploegh HL, Olsen BR, Mothes W. Secreted cathepsin L generates endostatin from collagen XVIII. EMBO J 2000; 19:1187–1194.

Fert-Bober J, Leon H, Sawicka J, et al. Inhibiting matrix metalloproteinase-2 reduces protein release into coronary effluent from isolated rat hearts during ischemia-reperfusion. Basic Res Cardiol 2008; 103:431-443.

Fiolet J, Baartscheer A, Schumacher C, Coronel R, Ter Welle H. The change of the free energy of ATP hydrolysis during global ischemia and anoxia in the rat hearts: its possible role in the regulation of transsarcolemmal sodium and potassium gradients. J Mol Cell Cardiol 1984; 16: 1023-1036.

Fliss H, Gattinger D. Apoptosis in ischemic and reperfused rat myocardium. Circ Res 1996; 79:949–956.

Frazier DP, Wilson A, Graham RM, Thompson JW, Bishopric NH, Webster KA. Acidosis regulates the stability, hydrophobicity, and activity of the BH3-only protein Bnip3. Antioxid Redox Signal 2006; 8:1625-1634.

French JP, Hamilton KL, Quindry JC, Lee Y, Upchurch PA, Powers SK. Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain. FASEB J 2008; 22:2862-2871.

French JP, Quindry JC, Falk DJ, et al. Ischemia-reperfusion-induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition. Am J Physiol Heart Circ Physiol 2006; 290:H128-H136.

Galli A, DeFelice LJ. Inactivation of L-type Ca^{2+} channels in embryonic chick ventricle cells: dependence on the cytoskeletal agents colchicine and taxol. Biophys J 1994; 67:2296-2304.

Ganote CE, Seabra-Gomes R, Nayler WG, Jennings RB. Irreversible myocardial injury in anoxic perfused rat hearts. Am J Pathol 1975; 80:419-450.

Gao WD, Atar D, Liu Y, Perez NG, Murphy AM, Marban E. Role of troponin I proteolysis in the pathogenesis of stunned myocardium. Circ Res 1997; 80:393–399.

Gilchrist JS, Cook T, Abrenica B, Rashidkhani B, Pierce GN. Extensive autolytic fragmentation of membranous versus cytosolic calpain following myocardial ischemia-reperfusion. Can J Physiol Pharmacol 2010; 88:584-594.

Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. Trends Biochem Sci 2007; 32:37-43.

González A, López B, Querejeta R, Zubillaga E, Echeverría T, Díez J. Filling pressures and collagen metabolism in hypertensive patients with heart failure and normal ejection

fraction. Hypertension 2010; 55:1418-1424.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994; 94:1621–1628.

Granzier HL, Labeit S. The giant protein titin: a major player in myocardial mechanics, signaling, and disease. Circ Res 2004; 94:284–295.

Gross J, Lapiere CM. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci USA 1962; 48:1014-1022.

Harris AS, Morrow JS. Proteolytic processing of human brain α-spectrin (fodrin): identification of a hypersensitive site. J Neurosci 1988; 8:2640-2651.

Hearse DJ, Humphrey SM, Chain EB. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: A study of myocardial enzyme release. J Mol Cell Cardiol 1973; 5:395-407.

Helske S, Syväranta S, Lindstedt KA, et al. Increased expression of elastolytic cathepsins S, K, and V and their inhibitory cystatin C in stenotic aortic valves. Arterioscler Thromb Vasc Biol 2006; 26:1791-1798.

Henry PD, Schuchleib R, Davis J, Weiss ES, Sobel BE. Myocardial contracture and accumulation of mitochondrial calcium in ischemic rabbit heart. Am J Physiol 1977; 233: H677-H684.

Hernando V, Inserte J, Sartório CL, Parra VM, Poncelas-Nozal M, Garcia-Dorado D. Calpain translocation and activation as pharmacological targets during myocardial ischemia/reperfusion. J Mol Cell Cardiol 2010; 49:271-279.

Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylaminde gels containing sodium dodecyl sulphate and colpolymerized substrates. Anal Biochem 1980; 102: 196-202.

Hobeika MJ, Edlin RS, Muhs BE, Sadek M, Gagne PJ. Matrix metalloproteinases in critical limb ischemia. J Surg Res 2008; 149:148-154.

Holmbeck K, Bianco P, Caterina J, et al. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell 1999; 99:81–92.

Holmbeck K, Bianco P, Yamada S, Birkedal-Hansen H. MT1-MMP: A tethered collagenase. J Cell Physiol 2004; 200:11–19.

Hooper NM, Hryszko J, Oppong SY, Turner AJ. Inhibition by converting enzyme inhibitors of pig kidney aminopeptidase P. Hypertension 1992; 19:281−285.

Horiuchi H, Suzuki T, Taniguchi M, et al. Attenuation of hepatic ischemia and reperfusion injury by serine protease inhibitor, FUT-175, in dogs. Transplant Proc 2001; 33:848.

Huang Y, Wang KK. The calpain family and human disease. Trends Mol Med 2001; 7:355-362.

Huang W, Wang Y, Askari A, Zolotarjova N, Ganjeizadeh M. Different sensitivies of the Na^+/K^+-ATPase isoforms to oxidants. Biochim Biophys Acta 1994; 1190: 108-114.

Inserte J, Barba I, Hernando V, Garcia-Dorado D. Delayed recovery of intracellular acidosis during reperfusion prevents calpain activation and determines protection in postconditioned myocardium. Cardiovasc Res 2009; 81:116-122.

Iwamoto H, Miura T, Okamura T, et al. Calpain inhibitor-1 reduces infarct size and DNA fragmentation of myocardium in ischemic/reperfused rat heart. J Cardiovasc Pharmacol 1999; 33:580-586.

Jane-Lise S, Corda S, Chassagne C, Rappaport L. The extracellular matrix and the cytoskeleton in heart hypertrophy and failure. Heart Fail Rev 2000; 5:239-250.

Juhaszova M, Blaustein MP. Na^+ pump low and high ouabain affinity alpha subunit isoforms are differently distributed in cells. Proc Natl Acad Sci USA 1997; 94:1800-1805.

Kandasamy AD, Chow AK, Ali MA, Schulz R. Matrix metalloproteinase-2 and myocardial oxidative stress injury: beyond the matrix. Cardiovasc Res 2010; 85:413-423.

Kandasamy AD, Schulz R. Glycogen synthase kinase-3β is activated by matrix metalloproteinase-2 mediated proteolysis in cardiomyoblasts. Cardiovasc Res 2009; 83:698-706.

Kar P, Samanta K, Shaikh S, Chowdhury A, Chakraborti T, Chakraborti S. Mitochondrial calpain system: an overview. Arch Biochem Biophys 2010; 495:1-7.

Kang PM, Haunstetter A, Aoki H, Usheva A, Izumo S. Morphological and molecular characterization of adult cardiomyocyte apoptosis during hypoxia and reoxygenation. Circ Res 2000; 87:118–125.

Kanoh M, Takemura G, Misao J, et al. Significance of myocytes with positive DNA *in situ* nick end-labeling (TUNEL) in hearts with dilated cardiomyopathy: Not apoptosis but DNA repair. Circulation 1999; 99:2757–2764.

Kawabata KI, Netticadan T, Osada M, Tamura K, Dhalla NS. Mechanisms of ischemic preconditioning effects on Ca^{2+} paradox-induced changes in heart. Am J Physiol Heart Circ Physiol 2000; 278:H1008–H1015.

Kher A, Meldrum KK, Hile KL, et al. Aprotinin improves kidney function and decreases tubular cell apoptosis and proapoptotic signaling after renal ischemia-reperfusion. J Thorac Cardiovasc Surg 2005; 130:662-669.

Kitakaze M, Weisfeldt ML, Marban E. Acidosis during early reperfusion prevents myocardial stunning in perfused ferret hearts. J Clin Invest 1988; 82:920-927.

Kleber A. Resting membrane potential, extracellular potassium activity, and intracellular sodium activity during acute global ischemia in isolated perfused guinea pig hearts. Circ Res 1983; 52: 442-450.

Kobayashi S, Yoshikawa Y, Sakata S, et al. Left ventricular mechanoenergetics after hyperpolarized cardioplegic arrest by nicorandil and after depolarized cardioplegic arrest by KCl. Am J Physiol Heart Circ Physiol 2004; 287:H1072-H1080.

Kostin S, Pool L, Elsasser A, et al. Myocytes die by multiple mechanisms in failing human hearts. Circ Res 2003; 92:715–724.

Kusuoka H, Porterfield JK, Weisman HF, Weisfeldt ML, Marban E.. Pathophysiology and pathogenesis of stunned myocardium. Depressed Ca^{2+} activation of contraction as a consequence of reperfusion-induced cellular Ca^{2+} overload in ferret hearts. J Clin Invest 1987; 79:950-961.

Kuster GM, Lancel S, Zhang J, et al. Redox-mediated reciprocal regulation of SERCA and Na^{+}-Ca^{2+} exchanger contributes to sarcoplasmic reticulum Ca^{2+} depletion in cardiac myocytes. Free Radic Biol Med 2010; 48:1182-1187.

Kyoi S, Otani H, Sumida T, et al. Loss of intracellular dystrophin: a potential mechanism for myocardial reperfusion injury. Circ J 2003; 67:725–727.

Lalu MM, Pasini E, Schulze CJ, et al. Ischaemia-reperfusion injury activates matrix metalloproteinases in the human heart. Eur Heart J 2005; 26:27-35.

Larsen TH, Dalen H, Sommer JR, Boyle R, Lieberman M. Membrane skeleton in cultured chick cardiac myocytes revealed by high resolution immunocytochemistry. Histochem Cell Biol 1999; 112:307-316.

Lee AA, McCulloch AD. Multiaxial myocardial mechanics and extracellular matrix remodeling: mechanochemical regulation of cardiac fibroblast function. Adv Exp Med Biol 1997; 430:227–240.

Lim CC, Zuppinger C, Guo X, et al. Anthracyclines induce calpain-dependent titin proteolysis and necrosis in cardiomyocytes. J Biol Chem 2004; 279:8290–8299.

Liu YH, Yang XP, Sharov VG, Sigmon DH, Sabbah HN, Carretero OA. Paracrine systems in the cardioprotective effect of angiotensin-converting enzyme inhibitors on myocardial ischemia/reperfusion injury in rats. Hypertension 1996; 27:7–13.

Lu L, Gunja-Smith Z, Woessner JF et al. Matrix metalloproteinases and collagen ultrastructure in moderate myocardial ischemia and reperfusion in vivo. Am J Physiol Heart Circ Physiol 2000; 279:H601-H609.

Luciani GB, D'Agnolo A, Mazzucco A, Gallucci V, Salviati G. Effects of ischemia on sarcoplasmic reticulum and contractile myofilament activity in human myocardium. Am J Physiol 1993; 265:H1334–H1341.

Ma H, Fukiage C, Kim YH, et al. Characterization and expression of calpain 10. A novel ubiquitous calpain with nuclear localization. J Biol Chem 2001; 276:28525-28531.

Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol Rev 2001; 53:245–282.

Maciewicz RA, Etherington DJ. A comparison of four cathepsins (B, L, N and S) with collagenolytic activity from rabbit spleen. Biochem J 1988; 256:433–440.

Maggiora LL, Orawski AT, Simmons WH. Apstatin analogue inhibitors of aminopeptidase P, a bradykinin-degrading enzyme. J Med Chem 1999; 42:2394-2402.

Makazan Z, Saini HK, Dhalla NS. Role of oxidative stress in alterations of mitochondrial function in ischemic-reperfused hearts. Am J Physiol Heart Circ Physiol 2007; 292: H1986-H1994.

Mani SK, Balasubramanian S, Zavadzkas JA, et al. Calpain inhibition preserves myocardial structure and function following myocardial infarction. Am J Physiol Heart Circ Physiol 2009; 297:H1744-H1751.

Marfella R, Di Filippo C, Portoghese M, et al. The ubiquitin-proteasome system contributes to the inflammatory injury in ischemic diabetic myocardium: the role of glycemic control. Cardiovasc Pathol 2009; 18:332-345.

Martinez-Zaguilan R, Seftor EA, Seftor REB, Chu YW, Gillies RJ, Hendrix MJ. Acidic pH enhances the invasive behavior of human melanoma cells. Clin Exp Metastasis 1996: 14;176-186.

Martorana PA, Kettenbach B, Breipohl G, Linz W, Schölkens BA. Reduction in infarct size by local angiotensin converting enzyme inhibition is abolished by a bradykinin antagonist. Eur J Pharmacol 1990; 182:395–396.

Massoudy P, Becker BF, Gerlach E. Nitric oxide accounts for postischemic cardioprotection resulting from angiotensin-converting enzyme inhibition: indirect evidence for a radical scavenger effect in isolated guinea pig heart. J Cardiovasc Pharmacol 1995; 25:440–447.

Massova I, Kotraa LP, Fridmanb R, Mobasherya S. Matrix metalloproteinases: structures, evolution, and diversification. FASEB J 1998; 12:1075-1095.

Matsumura Y, Kusuoka H, Inoue M, Hori M, Kamada T. Protective effect of the protease inhibitor leupeptin against myocardial stunning. J Cardiovasc Pharmacol 1993; 22:135-142.

McCallister LP, Munger BL, Neely JR. Electron microscopic observations and acid phosphatase activity in the ischemic rat heart. J Mol Cell Cardiol 1977; 9:353-364.

McDonald, JK. An overview of protease specificity and catalytic mechanisms: aspects related to nomenclature and classification. Histochem J 1985; 17:773-785.

Miner EC, Miller WL. A look between the cardiomyocytes: the extracellular matrix in heart failure. Mayo Clin Proc 2006; 81:71–76.

Mishra PK, Metreveli N, Tyagi SC. MMP-9 gene ablation and TIMP-4 mitigate PAR-1-mediated cardiomyocyte dysfunction: a plausible role of dicer and mRNA. Cell Biochem Biophys 2010; 57:67-76.

Mitani A, Shattock M. Role of Na-activated K channel, Na-K-Cl cotransport, and Na-K pump in $[K]_e$ changes during ischemia in rat heart. Am J Physiol Heart Circ Physiol 1992; 263: H333-H340.

Miura T. Adenosine and bradykinin: are they independent triggers of preconditioning? Basic Res Cardiol 1996; 9:20–22.

Mort JS, Buttle DJ. Cathepsin B. Int J Biochem Cell Biol 1997; 29:715-720.

Müller AL, Dhalla NS. Role of various proteases in cardiac remodeling and progression of heart failure. Heart Fail Rev 2011; 17: 395-409.

Müller AL, Freed D, Hryshko LV, Dhalla NS. Implications of protease activation in cardiac dysfunction and development of genetic cardiomyopathy in hamsters. Can J Physiol Pharmacol 2012a; [Epub ahead of print]

Müller AL, Hryshko LV, Dhalla NS. Extracellular and intracellular proteases in cardiac dysfunction due to ischemia-reperfusion injury. Int J Cardiol 2012b; [Epub ahead of print]

Munakata M, Stamm C, Friehs I, et al. Protective effects of protein kinase C during myocardial ischemia require activation of phosphatidyl-inositol specific phospholipase C. Ann Thorac Surg 2002; 73:1236–1245.

Nakamura M, Sunagawa M, Kosugi T, Sperelakis N. Actin filament disruption inhibits L-type Ca^{2+} channel current in cultured vascular smooth muscle cells. Am J Physiol Cell Physiol 2000; 279:C480-C487.

Naitoh M, Suzuki H, Arakawa K, Matsumoto A. Role of kinin and renal ANG II blockade in acute effects of ACE inhibitors in low renin-hypertension. Am J Physiol 1997; 272:H679–H687.

Narula J, Haider N, Virmani R, et al. Apoptosis in myocytes in end-stage heart failure. New Engl J Med 1996; 335:1182–1189.

Narula J, Pandey P, Arbustini E, et al. Apoptosis in heart failure: Release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc Natl Acad Sci USA 1999: 96:8144–8149.

Nath R, Raser KJ, Safford D. Non-erythroid α-spectrin breakdown by calpain and interleukin 1 β-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. Biochem J 1996; 319:683-690.

Neely JR, Grotyohann LW. Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. Circ Res 1984: 55;816-824.

Ong SB, Hausenloy DJ. Mitochondrial morphology and cardiovascular disease. Cardiovasc Res 2010; 88:16-29.

Orawski AT, Surz JP, Simmons WH. Metabolism of bradykinin by multiple coexisting membrane-bound peptidases in lung: techniques for investigating the role of each peptidase using specific inhibitors. Adv Exp Med Biol 1989; 247B:355–364.

Osada M, Netticadan T, Tamura K, Dhalla NS. Modification of ischemia-reperfusion-induced changes in cardiac sarcoplasmic reticulum by preconditioning. Am J Physiol Heart Circ Physiol 1998; 274:H2025–H2034.

Ostadal P, Elmoselhi AB, Zdobnicka I, Lukas A, Chapman D, Dhalla NS. Ischemia-reperfusion alters gene expression of Na+-K+ ATPase isoforms in rat heart. Biochem Biophys Res Commun 2003; 306:457–462.

Ostadal P, Elmoselhi AB, Zdobnicka I, Lukas A, Elimban V, Dhalla NS. Role of oxidative stress in ischemia-reperfusion-induced changes in Na+-K+-ATPase isoform expression in rat heart. Antioxid Redox Signal 2004; 6:914–923.

Papp Z, Van der Velden J, Stienen GJ. Calpain-I induced alterations in the cytoskeletal structure and impaired mechanical properties of single myocytes of rat heart. Cardiovasc Res 2000;45: 981-993.

Pedrozo Z, Sánchez G, Torrealba N, et al. Calpains and proteasomes mediate degradation of ryanodine receptors in a model of cardiac ischemic reperfusion. Biochim Biophys Acta 2010; 1802:356-362.

Penna C, Mancardi D, Rostaldo R, Paglioro P. Cardioprotection: a radical view – Free radicals in pre and postconditioning. Biochim Biophys Acta 2009; 1787:781-793.

Perrin BJ, Huttenlocher A. Calpain. Int J Biochem Cell Biol 2002; 34:722-725.

Peterson JT, Li H, Dillon L, Bryant JW. Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat. Cardiovasc Res 2000; 46:307-315.

Polewicz D, Cadete VJ, Doroszko A, et al. Ischemia induced peroxynitrite dependent modifications of cardiomyocyte MLC1 increases its degradation by MMP-2 leading to contractile dysfunction. J Cell Mol Med 2011; 15:1136-1147.

Powell SR, Wang P, Katzeff H, et al. Oxidized and ubiquitinated proteins may predict recovery of post-ischemic cardiac function: essential role of the proteasome. Antioxid Redox Signal 2005; 7:538–546.

Prechel MM, Orawski AT, Maggiora LL, Simmons WH. Effect of a new aminopeptidase P inhibitor, apstatin on bradykinin degradation in the rat lung. J Pharmacol Exp Ther 1995; 275:1136–1142.

Pruefer D, Buerke U, Khalil M, et al. Cardioprotective effects of the serine protease inhibitor aprotinin after regional ischemia and reperfusion on the beating heart. J Thorac Cardiovasc Surg 2002; 124:942-949.

Rajani V, Hussain Y, Bolla BS, et al. Attenuation of epinephrine-induced dysrhythmias by bradykinin: role of nitric oxide and prostaglandins. Am J Cardiol 1997; 80 (3A):153A–157A.

Randriamboavionjy V, Pistrosch F, Bölck B, et al. Platelet sarcoplasmic endoplasmic reticulum Ca2+-ATPase and mu-calpain activity are altered in type 2 diabetes mellitus and restored by rosiglitazone. Circulation 2008; 117:52-60.

Reiser J, Adair B, Reinheckel T. Specialized roles for cysteine cathepsins in health and disease. J Clin Invest 2010; 120:3421-3431.

Remme WJ. Bradykinin-mediated cardiovascular protective actions of ACE inhibitors. A new dimension in anti-ischemic therapy? Drugs 1997; 54 Suppl 5;59–70.

Remppis A, Scheffold T, Greten J, et al. Intracellular compartmentation of troponin T: release kinetics after global ischemia and calcium paradox in the isolated perfused rat heart. J Mol Cell Cardiol 1995; 27:793–803.

Rett K, Wicklmayr M, Dietze GJ. Metabolic effects of kinins: historical and recent developments. J Cardiovasc Pharmacol 1990; 15(Suppl 6):S57–S59.

Rodríguez D, Morrison CJ, Overall CM. Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta 2010; 1803:39-54.

Saini HK, Dhalla NS. Defective calcium handling in cardiomyocytes isolated from hearts subject3ed to ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2005; 288: H2260-H2270.

Saini HK, Elimban V, Dhalla NS. Attenuation of extracellular ATP response in cardiomyocytes isolated from hearts subjected to ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2005; 289: H614-H623.

Samanta K, Kar P, Chakraborti T, Chakraborti S. Calcium-dependent cleavage of the Na^{+}/Ca^{2+} exchanger by m-calpain in isolated endoplasmic reticulum. J Biochem 2010a; 147:225-235.

Samanta K, Kar P, Chakraborti T, Shaikh S, Chakraborti S. Characteristic properties of endoplasmic reticulum membrane m-calpain, calpastatin and lumen m-calpain: a comparative study between membrane and lumen m-calpains. J Biochem 2010b; 147:765-779.

Sauvé M, Ban K, Momen MA, et al. Genetic deletion or pharmacological inhibition of dipeptidyl peptidase-4 improves cardiovascular outcomes after myocardial infarction in mice. Diabetes 2010; 59:1063-1073.

Sawicki G, Leon H, Sawicka J, et al. Degradation of myosin light chain in isolated rat hearts subjected to ischemia–reperfusion injury: a new intracellular target for matrix metalloproteinase-2. Circulation 2005; 112:544–552.

Schenke-Layland K, Stock UA, Nsair A, et al. Cardiomyopathy is associated with structural remodelling of heart valve extracellular matrix. Eur Heart J 2009; 30:2254–2265.

Scholkens BA. Kinins in the cardiovascular system. Immunopharmacology 1996; 30:209–216.

Schoutsen B, Blom JJ, Verdouw PD, Lamers JM. Calcium transport and phospholamban in sarcoplasmic reticulum of ischemic myocardium. J Mol Cell Cardiol 1989; 21:719–727.

Schrör K. Role of prostaglandins in the cardiovascular effects of bradykinin and angiotensin-converting enzyme inhibitors. J Cardiovasc Pharmacol 1992; 20:S68–S73.

Schulz R. Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. Annu Rev Pharmacol Toxicol 2007; 47:211-242.

Schulze CJ, Wang W, Saurez-Pinzon WL, Sawicka J, Sawicka G, Schulz R. Imbalance between tissue inhibitor of metalloproteinase-4 and matrix metalloproteinases during acute myocardial ischemia-reperfusion injury. Circulation 2003; 107:2487-2492.

Schwartz SM, Duffy JY, Pearl JM, Goins S, Wagner CJ, Nelson DP. Glucocorticoids preserve calpastatin and troponin I during cardiopulmonary bypass in immature pigs. Pediatr Res 2003; 54:91-97.

Schwertz H, Carter JM, Russ M, et al. Serine protease inhibitor nafamostat given before reperfusion reduces inflammatory myocardial injury by complement and neutrophil inhibition. J Cardiovasc Pharmacol 2008; 52:151-160.

Shimada Y, Avkiran M. Attenuation of reperfusion arrhythmias by selective inhibition of angiotensin-converting enzyme/kininase II in the ischemic zone: mediated by endogenous bradykinin? J Cardiovasc Pharmacol 1996; 27:428–438.

Simmons G, Gosalia DN, Rennekamp AJ, et al. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. PNAS 2005; 102:11876-11881.

Simmons WH, Orawski AT. Membrane-bound aminopeptidase P from bovine lung: its purification, properties and degradation of bradykinin. J Biol Chem 267; 4:897–903.

Singh RB, Chohan PK, Dhalla NS, Netticadan T. The sarcoplasmic reticulum proteins are targets for calpain action in the ischemic-reperfused heart. J Mol Cell Cardiol 2004a; 37:101–110.

Singh RB, Dandekar SP, Elimban V, Gupta SK, Dhalla NS. Role of proteases in the pathophysiology of cardiac disease. Mol Cell Biochem 2004b; 263:241-56.

Singh RB, Elimban V, Dhalla NS. Differences in ischemia-reperfusion-induced endothelial changes in hearts perfused at constant flow and constant pressure. J Appl Physiol 2008; 105:1779-1787.

Singh RB, Dhalla NS. Ischemia-reperfusion-induced changes in sarcolemma Na^+-K^+-ATPase are due to the activation of calpain in the heart. Can J Physiol Pharmacol 2010; 88: 388-397.

Singh RB, Hryshko L, Freed D, Dhalla NS. Activation of proteolytic enzymes and depression of the sarcolemma Na^+-K^+-ATPase in ischemia-reperfused heart may be mediated through oxidative stress. Can J Physiol Pharmacol 2012; 90: 249-260.

Siragy HM. Evidence that intrarenal bradykinin plays a role in regulation of renal function. Am J Physiol 1993; 265:E648–E654.

Sivasubramanian N, Coker ML, Kurrelmeyer KM, et al. Left ventricular remodeling in transgenic mice with cardiac restricted overexpression of tumor necrosis factor. Circulation 2001; 104:826–831.

Sodhi RK, Singh M, Singh N, Jaggi AS. Protective effects of caspase-9 and poly (ADP-ribose) polymerase inhibitors on ischemia-reperfusion induced myocardial injury. Arch Pharm Res 2009; 32:1037-1043.

Sorimachi Y, Harada K, Saido TC, Ono T, Kawashima S, Yoshida K. Downregulation of calpastatin in rat heart after brief ischemia and reperfusion. J Biochem 1997; 122:743-748.

Spinale FG. Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. Physiol Rev 2007; 87:1285-1342

Spinale FG. Amplified bioactive signaling and proteolytic enzymes following ischemia reperfusion and aging: Remodeling pathways that are not like fine wine. Circulation 2010; 12:322-324.

Streeter DD Jr, Bassett DL. An engineering analysis of myocardial fiber orientation in pig's left ventricle in systole. Anat Rec 1966; 155:503–511

Streeter DD Jr, Spotnitz HM, Patel DP, Ross J Jr, Sonnenblick EH. Fiber orientation in the canine left ventricle during diastole and systole. Circ Res 1969; 24:339–347.

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. J Biol Chem 1995; 270:5331-5338.

Sung MM, Schulz CG, Wang W, Sawicki G, Bautista-Lopez NL, Schulz R. Matrix metalloproteinase-2 degrades the cytoskeletal protein alpha-actinin in peroxynitrite mediated myocardial injury. J Mol Cell Cardiol 2007; 43:429-436.

Swift F, Tovsrud N, Enger UH, Sjaastad I, Sejersted OM. The Na^+,K^+-ATPase α2-isoform regulates cardiac contractility in rat cardiomyocytes. Cardiovasc Res 2007; 75:109-117.

Sybers HD, Myre CD, Myre MV. X-ray microanalysis of calcium in ischemic myocardium: a new method of rapid freezing. Scan Electron Microsc 1983; 769-776.

Temsah RM, Netticadan T, Chapman D, Takeda S, Mochizuki S, Dhalla NS. Alterations in sarcoplasmic reticulum function and gene expression in ischemic-reperfused rat heart. Am J Physiol 1999; 277:H584–H594.

Terkildsen JR, Crampin EJ, Smith NP. The balance between inactivation and activation of the Na^+-K^+ pump underlies the triphasic accumulation of K^+ during myocardial ischemia. Am J Physiol Heart Circ Physiol 2007; 293:H3036-H3045.

Tiwari M, Hemalatha T, Ganesan K, et al. Myocardial ischemia and reperfusion injury in rats: lysosomal hydrolases and matrix metalloproteinases mediated cellular damage. Mol Cell Biochem 2008; 312:81-91.

Tskhovrebova L, Trinick J. Roles of titin in the structure and elasticity of the sarcomere. J Biomed Biotechnol 2010; 2010:612482.

Turk B, Turk V, Turk D. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. J Biol Chem 1997; 378:141-150.

Turner GA. Increased release of tumour cells by collagenase at acid pH: a possible mechanism for metastasis. Experimentia 1979; 35:1657-1658.

Turski WA, Zasłonka J. Activity of cathepsin D and L in the heart muscle of coronary patients during coronary-aortal bypass graft operation. Med Sci Monit 2000; 6:853-860.

Tsuji T, Ohga Y, Yoshikawa Y, et al. Rat cardiac contractile dysfunction induced by Ca^{2+} overload: possible link to the proteolysis of fodrin. Am J Physiol Heart Circ Physiol 2001; 281:H1286-H1294.

Urbich C, Heeschen C, Aicher A, et al. Cathepsin L is required for endothelial progenitor cell-induced neovascularization. Nat Med 2005; 11:206–213.

Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J Biol Chem 1998; 273:18092-18098.

Wang W, Schulze CJ, Suarez-Pinzon WL, et al. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. Circulation 2002; 106:1543-1549.

Webb SD, Sherratt JA, Fish RG. Modelling tumour acidity and invasion. Novartis Found Symp 2001; 240:169-181.

Westfall MV, Solaro RJ. Alterations in myofibrillar function and protein profiles after complete global ischemia in rat hearts. Circ Res 1992; 70:302–313.

Wildenthal K. Lysosomal alterations in ischemic myocardium: Result or cause of myocellular damage? J Mol Cell Cardiol 1978; 10:595-603.

Willis MS, Schisler JC, Portbury AL, Patterson C. Build it up-tear it down: protein quality control in the cardiac sarcomere. Cardiovasc Res 2009; 81:439-448.

Wilson EM, Spinale FG. Myocardial remodeling and matrix metalloproteinases in heart failure: turmoil within the interstitium. Ann Med 2001; 33:623-634.

Wolfrum S, Richardt G, Dominiak P, Katus HA, Dendorfer A. Apstatin, a selective inhibitor of aminopeptidase P, reduces myocardial infarct size by a kinin-dependent pathway. Br J Pharmacol 2001; 134:370-374.

Van Eyk JE, Powers F, Law W, Larue C, Hodges RS, Solaro RJ. Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: identification of degradation products and effects on the pCa-force relation. Circ Res 1998; 82:261–271.

Vanhoutte D, Schellings M, Pinto Y, Heymans S. Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. Cardiovasc Res 2006; 69:604-613.

Veeravalli KK, Akula A, Routhu KV, Kota MK. Infarct size limiting effect of apstatin alone and in combination with enalapril, lisinopril and ramipril in rats with experimental myocardial infarction. Pharmacol Res 2003; 48:557-563.

Yamamoto F, Yamamoto H, Yoshida S, et al. The effects of several pharmacologic agents upon postischemic recovery. Cardiovasc Drugs Ther 1991; 8:301–308.

Yarbrough WM, Mukherjee R, Stroud RE et al. Caspase inhibition modulates left ventricular remodeling following myocardial infarction through cellular and extracellular mechanisms. J Cardiovasc Pharmacol 2010; 55:408-416.

Yasuda Y, Li Z, Greenbaum D, Bogyo M, Weber E, Bromme D. Cathepsin V, a novel and potent elastolytic activity expressed in activated macrophages. J Biol Chem 2004; 279:36761–36770.

Yoshida K, Inui M, Harada K, et al. Reperfusion of rat heart after brief ischemia induces proteolysis of calspectin (nonerythroid spectrin or fodrin) by calpain. Circ Res 1995; 77:603–610.

Yoshida Y, Shiga T, Imai S. Degradation of sarcoplasmic reticulum calcium-pumping ATPase in ischemic-reperfused myocardium: role of calcium-activated neutral protease. Basic Res Cardiol 1990; 85:495–507.

Yoshikawa Y, Hagihara H, Ohga Y, et al. Calpain inhibitor-1 protects the rat heart from ischemia-reperfusion injury: analysis by mechanical work and energetics. Am J Physiol Heart Circ Physiol 2005; 288:H1690-H1698.

Yoshikawa Y, Zhang GX, Obata K, et al. Cardioprotective effects of a novel calpain inhibitor SNJ-1945 for reperfusion injury after cardioplegic cardiac arrest. Am J Physiol Heart Circ Physiol 2010; 298:H643-H651.

Yoshimoto T, Orawski AT, Simmons WH. Substrate specificity of aminopeptidase P from *Escherichia coli*: Comparison with membrane-bound forms from rat and bovine lung. Arch Biochem Biophys 1994; 311:28−34.

Ytrehus K, Myklebust R, Olsen R, Mjos OD. Ultrastructural changes induced in the isolated rat heart by enzymatically generated oxygen radicals. J Mol Cell Cardiol 1987;19:379-389.

Zahler R, Gilmore-Herbert M, Baldwin JC, Franco K, Benz EJ Jr. Expression of alpha isoforms of the Na,K-ATPase in human heart. Biochim Biophys Acta 1993; 1149:189-194.

Zaidi N, Maurer A, Nieke S, Kalbacher H. Cathepsin D: a cellular roadmap. Biochem Biophys Res Commun 2008; 376:5-9.

Zaugg M, Schaub MC. Signaling and cellular mechanisms in cardiac protection by ischemic and pharmacological preconditioning. J Muscle Res Cell Motil 2004; 25:219-249.

Zhou HZ, Ma X, Gray MO, et al. Transgenic MMP-2 expression induces latent cardiac mitochondrial dysfunction. Biochem Biophys Res Commun 2007; 358:189-195.

Zhao ZQ, Nakamura M, Wang NP, et al. Reperfusion induces myocardial apoptotic cell death. Cardiovasc Res 2000; 45:651–660.

Zucchi R, Ronca F, Ronca-Testoni S. Modulation of sarcoplasmic reticulum function: a new strategy in cardioprotection? Pharmacol Ther 2001; 89:47–65.

Zucchi R, Ronca-Testoni S, Yu G, Galbani P, Ronca G, Mariani M. Effect of ischemia and reperfusion on cardiac ryanodine receptors – sarcoplasmic reticulum Ca^{2+} channels. Circ Res 1994; 74:271–280.